Supplementary Information

mRNA cap analogues substituted in the tetraphosphate chain with CX_2: identification of O-to-CCl_2 as the first bridging modification that confers resistance to decapping without impairing translation

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Contents

Supplementary figures and tables

Table 1 pK_a values of mononucleotide analogues **2a-2d** as determined by ^{31}P NMR assay.

compound	concentration [mM]	pK_a^4 (gamma)*	pK_a^4 (beta)**	Mean pK_a^4
m^7Gppp/NH_4^+ (**2d**)	15.0	6.55±0.02	6.62±0.04	6.56±0.03
m^7GppCH_2p/Na^+ (**2a**)	5.49	8.63±0.02	8.63±0.01	8.63±0.01
$m^7GppCCl_2p/NH_4^+$ (**2b**)	5.33	7.39±0.02	7.25±0.02	7.32±0.07
m^7GppCF_2p/NH_4^+ (**2c**)	7.50	6.21±0.02	6.29±0.03	6.25±0.04

* pK_a^4 as determined using change of chemical shift for gamma-phosphate.

** pK_a^4 as determined using change of chemical shift for beta-phosphate.

pK_a^4 using change of chemical shift for alpha-phosphates was not determined due to only small changes in chemical shifts for alpha-phosphates upon pH change. The mean pK_a^4 values were determined as weighted mean of pK_a^4 (gamma) and pK_a^4 (beta) values.

The collected data were fitted to DoseResp function in OriginLab software using fallowing equation:

$$chemical\ shift = A_1 + \frac{(A_2 - A_1)}{1 + 10^{(pKa - pH)*p}} \quad (1_a)$$

where the $\mathbf{A_1}$ and $\mathbf{A_2}$ are bottom and top asymptote respectively, **p** is the hill slope. The pK_a values were determined for both gamma and beta phosphate and the weighted average with uncertainties were calculated using equations $2_{a\text{-}c}$.

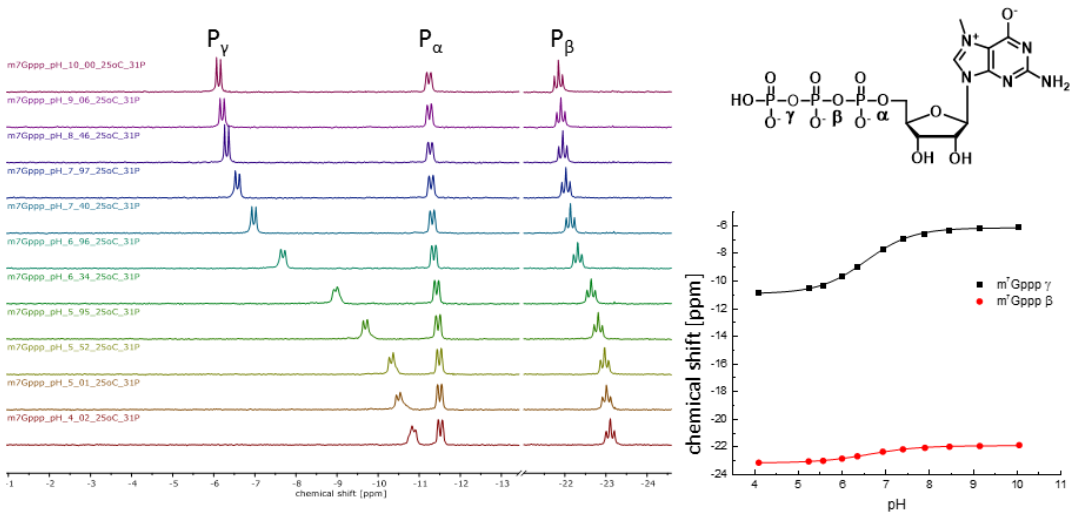
$$\bar{x}_w = \frac{\sum_{i=1}^{N} \frac{x_i}{u_i^2}}{\sum_{i=1}^{N} \frac{1}{u_i^2}} \quad (2_a)$$

$$u_{int} = \sqrt{\sum_{i=1}^{N} \frac{1}{u_i^2}} \quad (2_b)$$

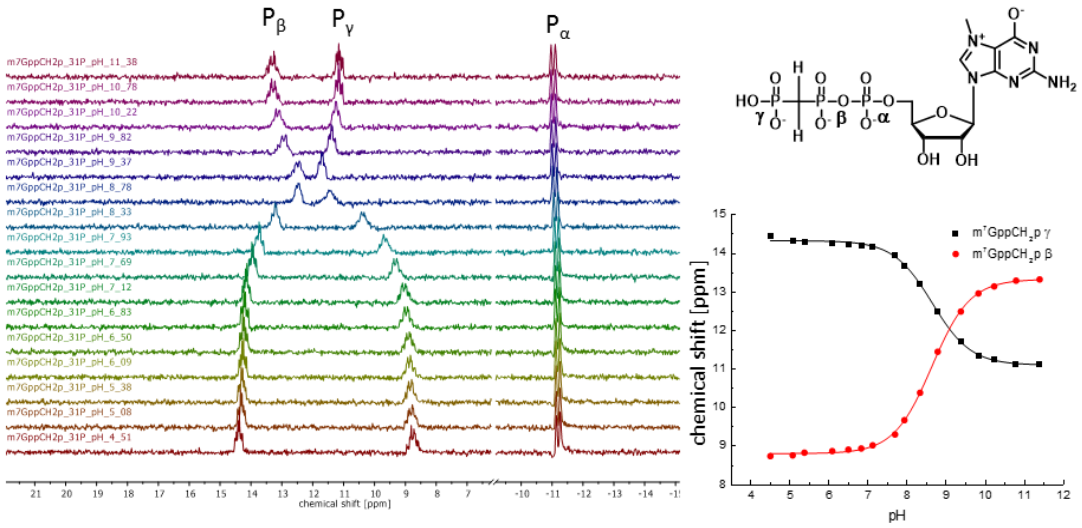
$$u_{ext} = \sqrt{\frac{u_{int}^2}{N-1} \sum_{i=1}^{N} \left(\frac{x_i - \bar{x}_w}{u_i}\right)} \quad (2_c)$$

Fig. S1 Titrations of cap analogues **2a-2d** as monitored by ^{31}P NMR. The NMR spectra and titration curves for analogues **2d** (**A**), **2a** (**B**), **2b** (**C**) and **2c** (**D**).

A



B



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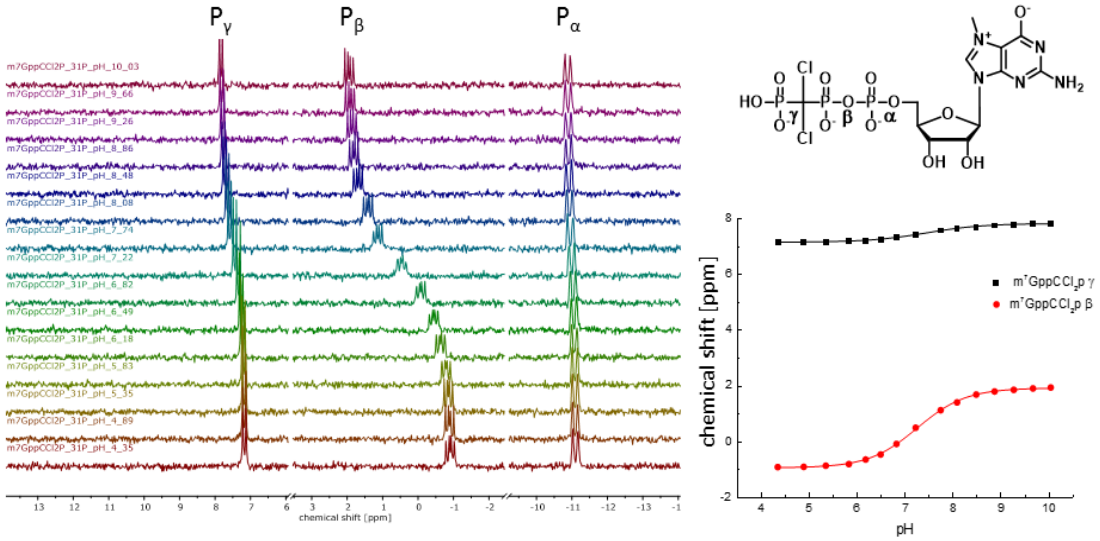

Pγ
Pβ
Pα
chemical shift [ppm]
chemical shift [ppm]
pH
m7GppCCl2p γ
m7GppCCl2p β

D

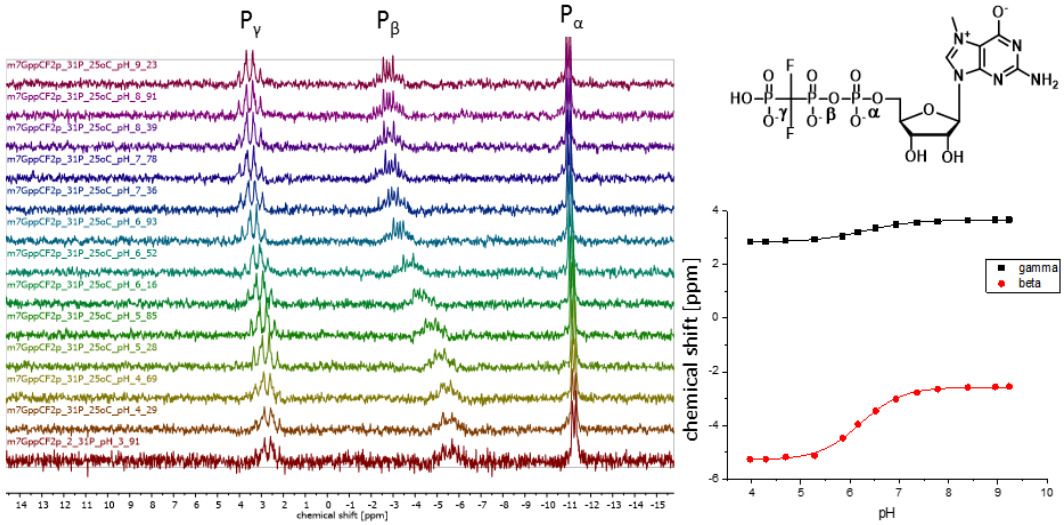

Pγ
Pβ
Pα
chemical shift [ppm]
chemical shift [ppm]
pH
gamma
beta

Table S2 Comparison for pK_a^4 values of mononucleoside triphosphates.

compound	pK_a^4 value*	compound	pK_a^4 value	compound	pK_a^4 value
Appp	7.1	Gppp	n.d.	m^7Gppp	6.56±0.03
AppCH_2p	8.4	GppCH_2p	n.d.	m^7GppCH_2p	8.63±0.01
AppCCl_2p	7.0	GppCCl_2p	7.49±0.03	m^7GppCCl_2p	7.32±0.07
AppCF_2p	7.1	GppCF_2p	6.51±0.07	m^7GppCF_2p	6.25±0.04

*Data from Blackburn et al.[1]

Fig. S2 Titrations of $GppCCl_2p$ (**4b**) and $GppCF_2p$ (**4c**) as monitored by ^{31}P NMR. The NMR spectra and titration curves for analogues **4b** (**A**) and **4c** (**B**).

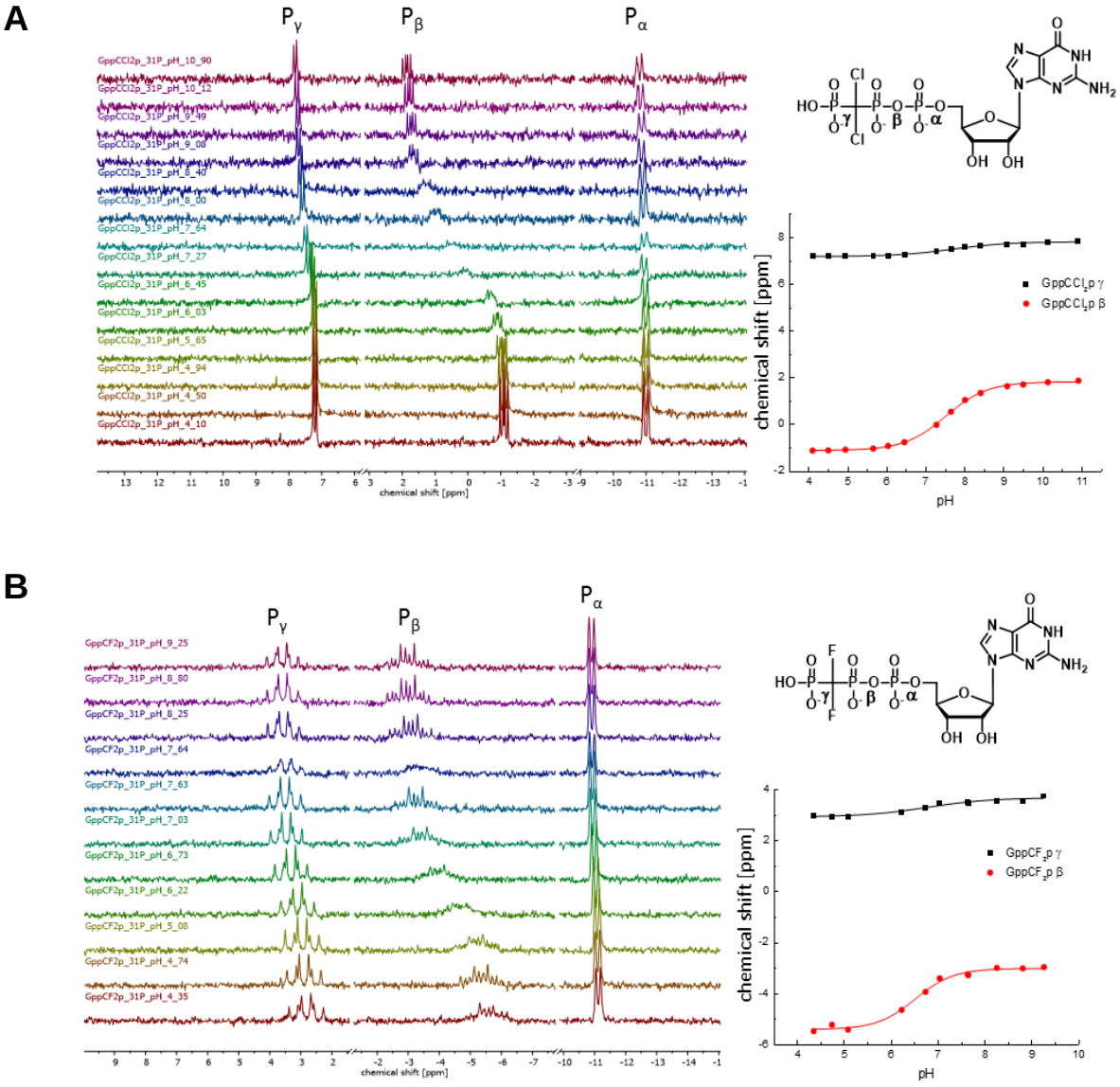


Fig. S3 Fluorescence titration curves for binding of cap analogues **2b-c** and **6b-c** to eIF4E.

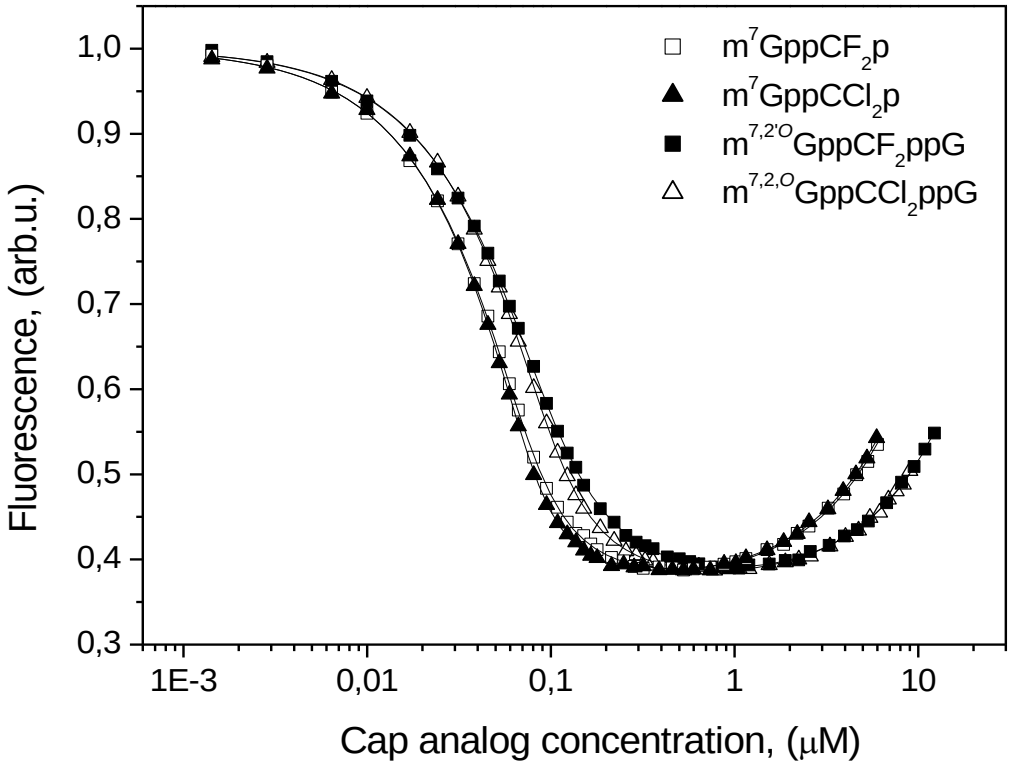


Fig. S4 DcpS and Dcp2 catalysed hydrolysis of various nucleotide substrates.

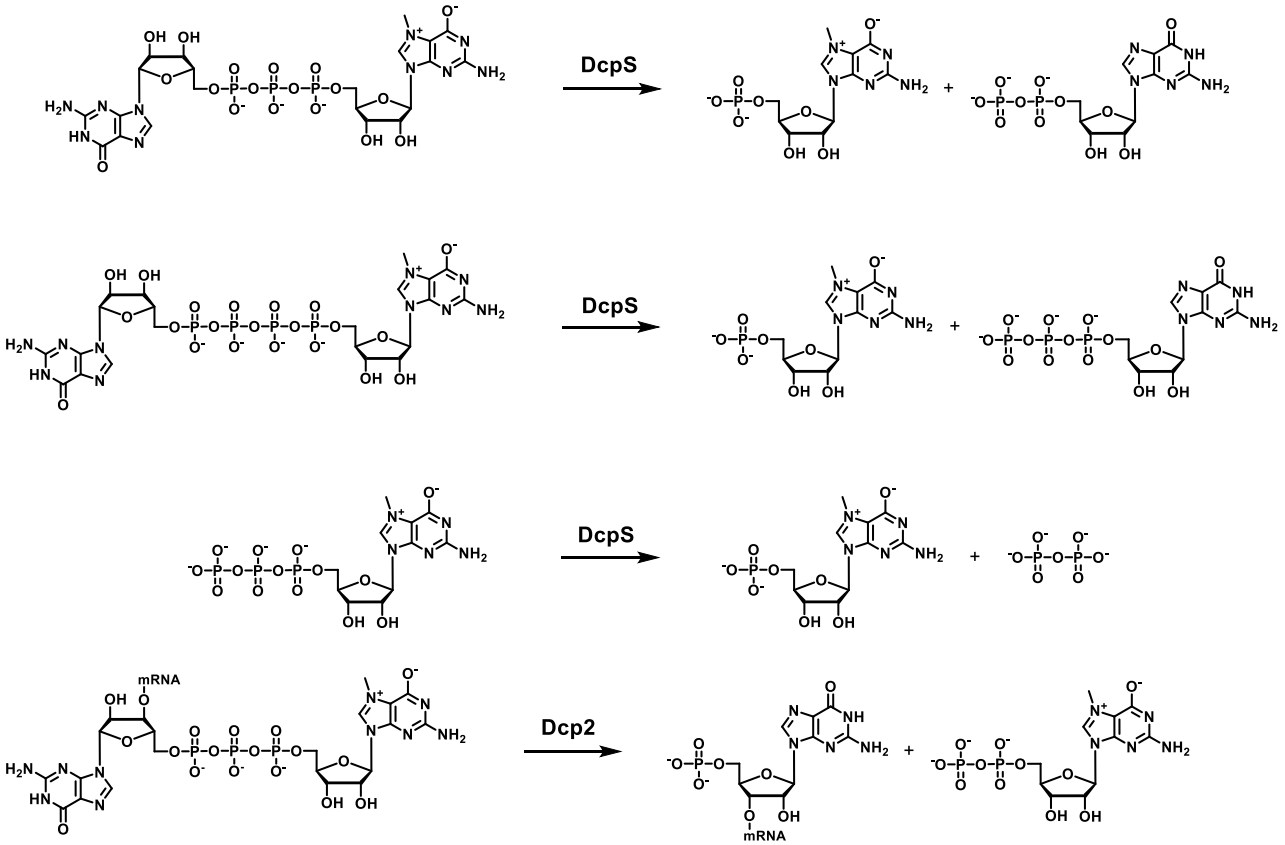


Table S3 Hydrolysis of cap analogues by human DcpS as monitored by HPLC. Data are average from duplicate experiments.

Cap analogue	% hydrolysis in given time			
	15 min	30 min	60 min	120 min
m^7GpppG	67.0 ± 7.0	95.1 ± 3.0	98.2 ± 1.8	100 ± 0
m^7Gppp (**2d**)	12.1 ± 0.9	22.3 ± 1.6	43.9 ± 3.5	77.6 ± 1.6
m^7GppCH_2p (**2a**)	0	0	0	0
$m^7GppCCl_2p$ (**2b**)	0.2 ± 0.2	0.7 ± 0.0	1.1 ± 0.3	1.7 ± 0.7
m^7GppCF_2p (**2c**)	2.9 ± 0.2	4.0 ± 0.8	6.9 ± 0.0	14.4 ± 0.6
m^7GppppG (**6d**)	31.0 ± 0.8	55.7 ± 0.8	90.0 ± 2.3	100 ± 0
m^7GppCH_2ppG (**6a**)	0	0	0	0
$m^7GppCCl_2ppG$ (**6b**)	0	0	0	0
m^7GppCF_2ppG (**6c**)	13.7 ± 0.2	26.2 ± 0.4	50.1 ± 1.8	87.5 ± 0.4
$m_2^{7,2'\text{-}O}GppCCl_2ppG$ (**7b**)	0	0	0	0
$m_2^{7,2'\text{-}O}GppCF_2ppG$ (**7c**)	2.3 ± 0.4	4.7 ± 0.2	8.1 ± 0.7	15.7 ± 1.9

$m_2^{7,2'\text{-}O}GppCH_2ppG$ (**7a**) was found to be resistant towards enzymatic hydrolysis by hDcpS in a similar assay conditions.[2] Error corresponds to standard deviation of two independent experimental points.

Fig. S5 Time course of DcpS mediated hydrolysis of modified cap analogues. Data are average from duplicate experiments.

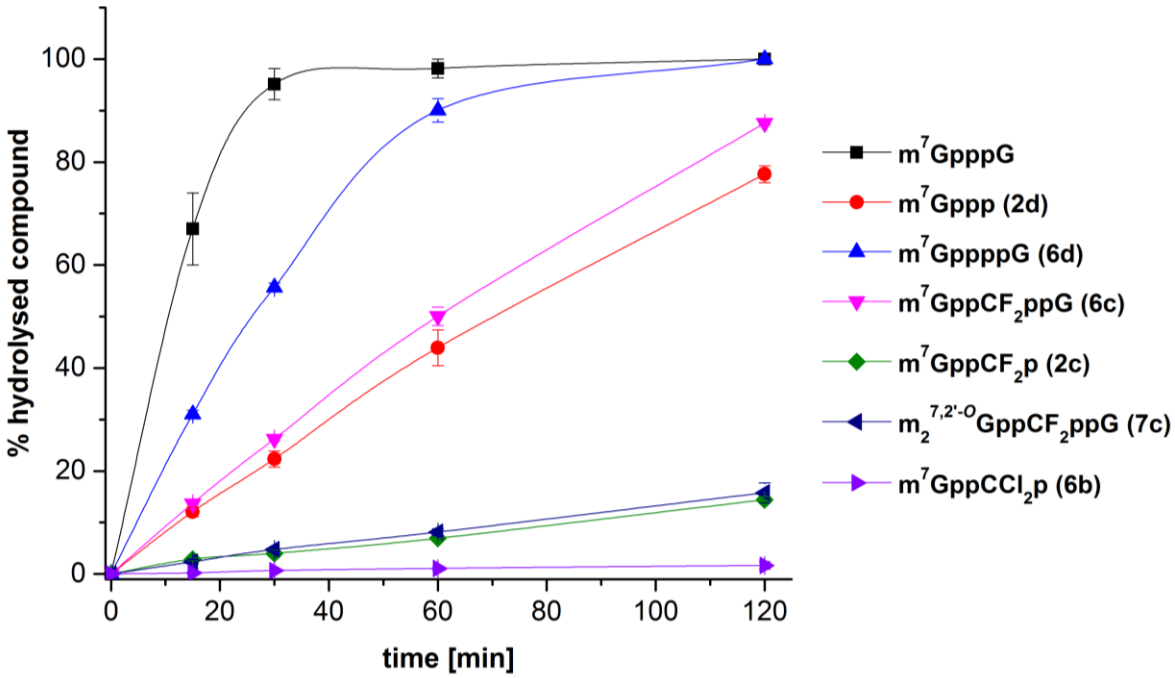


Fig. S6 Capping efficiencies (A) and susceptibilities (B) of 26-nt transcripts capped with various cap analogs to the hDcp2. Reactions were terminated at the indicated time points followed by denaturing PAGE, stained with SYBR Gold and visualized. Figure presents representative result of one biological repetition.

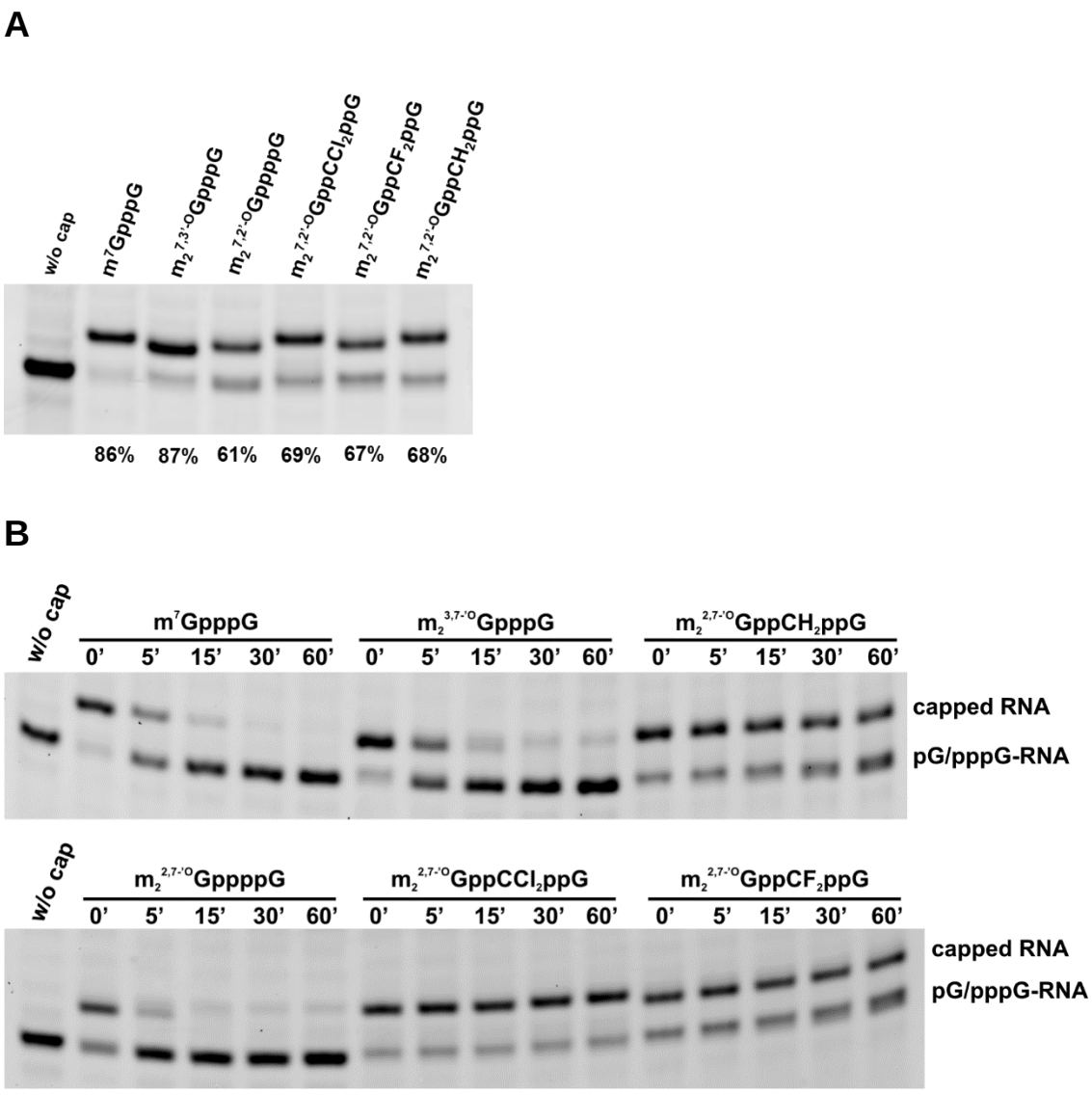
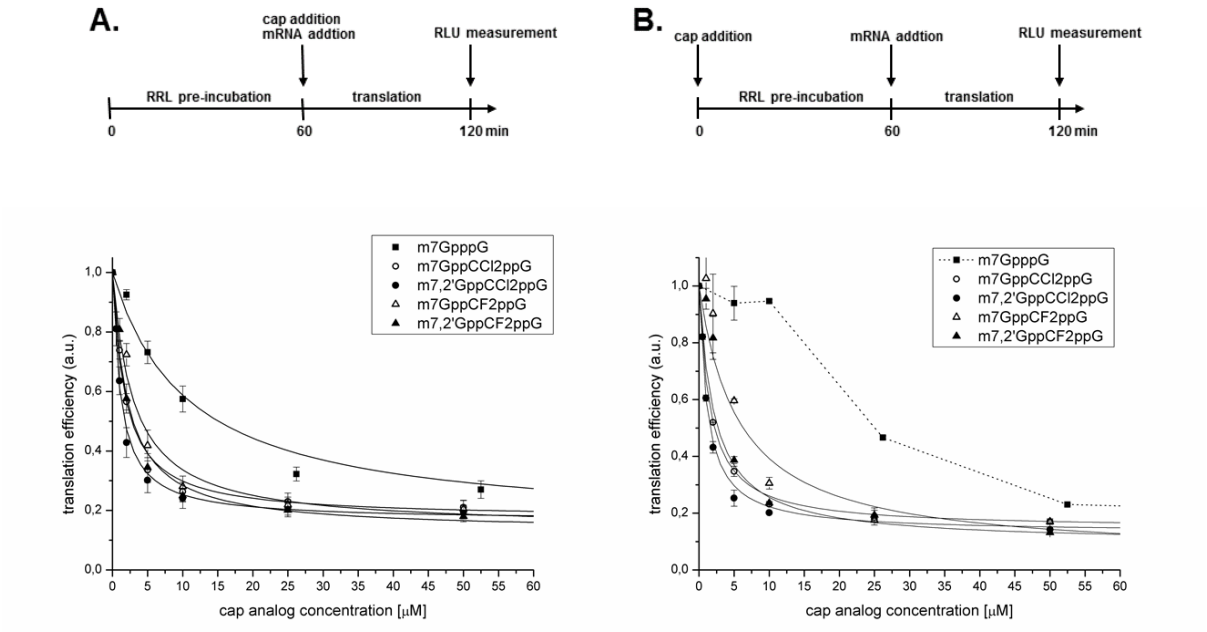
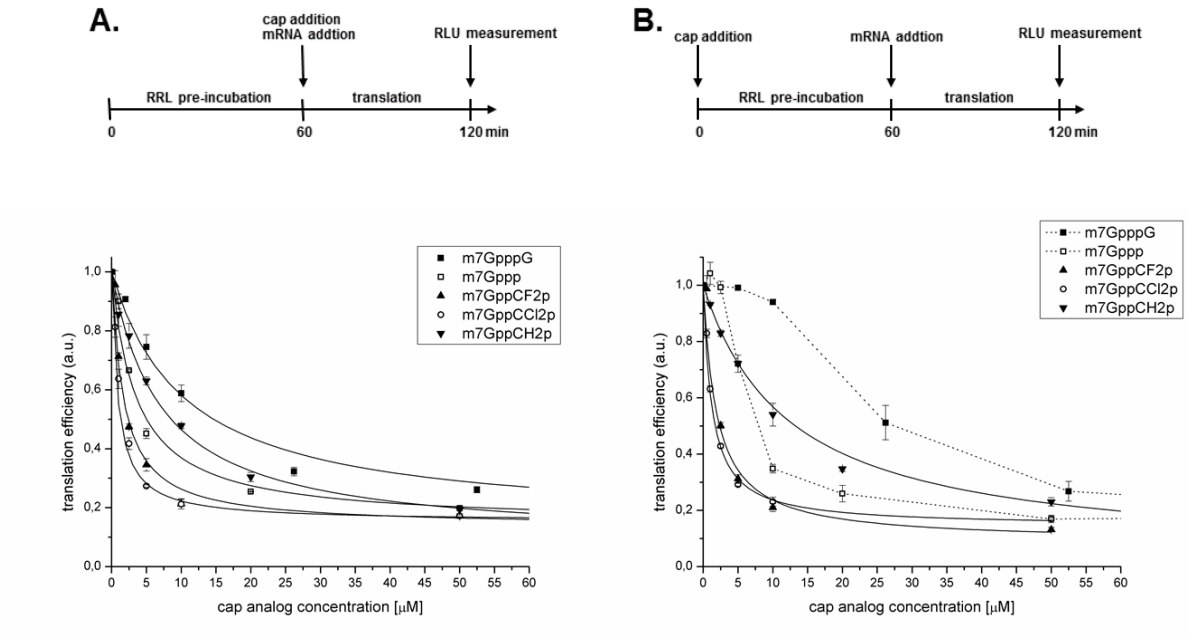


Fig. S7 Inhibition of translation of $m_2^{7,3'\text{-}O}$GpppG-capped luciferase encoding mRNA in rabbit reticulocyte lysate (RRL) by dinucleotide cap analogues.



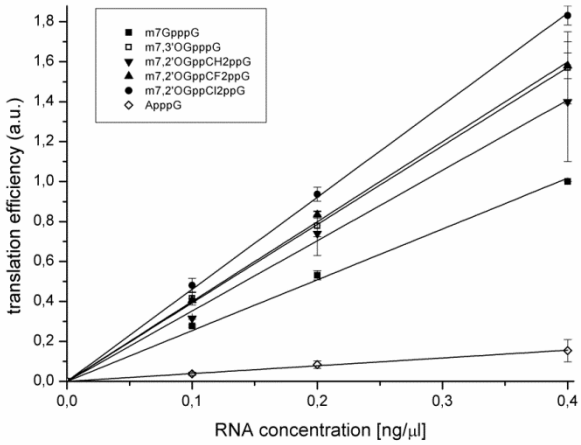
Inhibition of translation of $m_2^{7,3'\text{-}O}$GpppG-capped luciferase encoding mRNA in rabbit reticulocyte lysate (RRL) by m^7GpppG, m^7GppCF_2pG, $m_2^{7,2'\text{-}O}GppCF_2pG$, m^7GppCl_2pG and $m_2^{7,2'\text{-}O}GppCl_2pG$. In experiment (A) the cap analogue and luciferase mRNA were added to RRL at the same time point. In experiment (B), to test stability of presented here cap analogues in reticulocyte lysate, the cap analogue was preincubated for 1 hour in RRL prior to addition of mRNA and start of translation. As it is seen in graph (B), the inhibitory properties of unmodified m^7GpppG is significantly diminished upon incubation in RRL (dotted line). In both experiments the luciferase activity was measured after 1 hour after mRNA addition. In the figure are shown data of 3 independent inhibition experiments (±SE) and 2 stability experiments (±SE), normalized to the translation efficiency without cap analog.

Fig. S8 Inhibition of translation of $m_2^{7,3'\text{-}O}$GpppG-capped luciferase encoding mRNA in rabbit reticulocyte lysate (RRL) by mononucleotide cap analogues.



Inhibition of translation of $m_2^{7,3'\text{-}O}$GpppG-capped luciferase encoding mRNA in rabbit reticulocyte lysate (RRL) by m^7GpppG, m^7GTP, m^7GppCF_2p, m^7GppCl_2p and m^7GppCH_2p. In experiment (A) the cap analogue and luciferase mRNA were added to RRL at the same time point. In experiment (B), to test stability of presented here cap analogues in reticulocyte lysate, the cap analogue was preincubated for 1 hour in RRL prior to addition of mRNA and start of translation. As it is seen in graph (B), the inhibitory properties of unmodified m^7GpppG and m^7GTP are significantly diminished upon incubation in RRL (dotted line). In both experiments the luciferase activity was measured after 1 hour after mRNA addition. In the figure are shown data of 3 independent inhibition experiments (±SE) and 2 stability experiments (±SE), normalized to the translation efficiency without cap analog.

Fig. S9 Translation efficiencies of mRNA encoding firefly luciferase capped with cap analogues **7a-7c**.



Translation of mRNAs encoding firefly luciferase capped with $m_2^{7,2'\text{-}O}GppCF_2pG$, $m_2^{7,2'\text{-}O}GppCl_2pG$ and $m_2^{7,2'\text{-}O}GppCH_2pG$, and with standard caps: m^7GpppG and $m_2^{7,3'\text{-}O}GpppG$ (Anti Reverse Cap Analog). Activity of luciferase (in RLU) synthesized in rabbit reticulocyte lysate were normalized to the activity obtained with m^7GpppG-capped RNA at the highest concentration used. The mean of 3 independent translation experiments (± SE) and showed in the graph as a function of capped luciferase mRNA concentration (except $m_2^{7,2'\text{-}O}GppCF_2pG$ with 2 experiments). A transcript capped with non-functional ApppG dinucleotide was added as a control of cap-dependent translation in RRL. After linear fitting to the experimental data points the slope values (of the linear regression equation) calculated for the differentially capped luciferase RNAs were compared to the slope value obtained for m^7GpppG-capped luciferase RNA that was set as = 1.

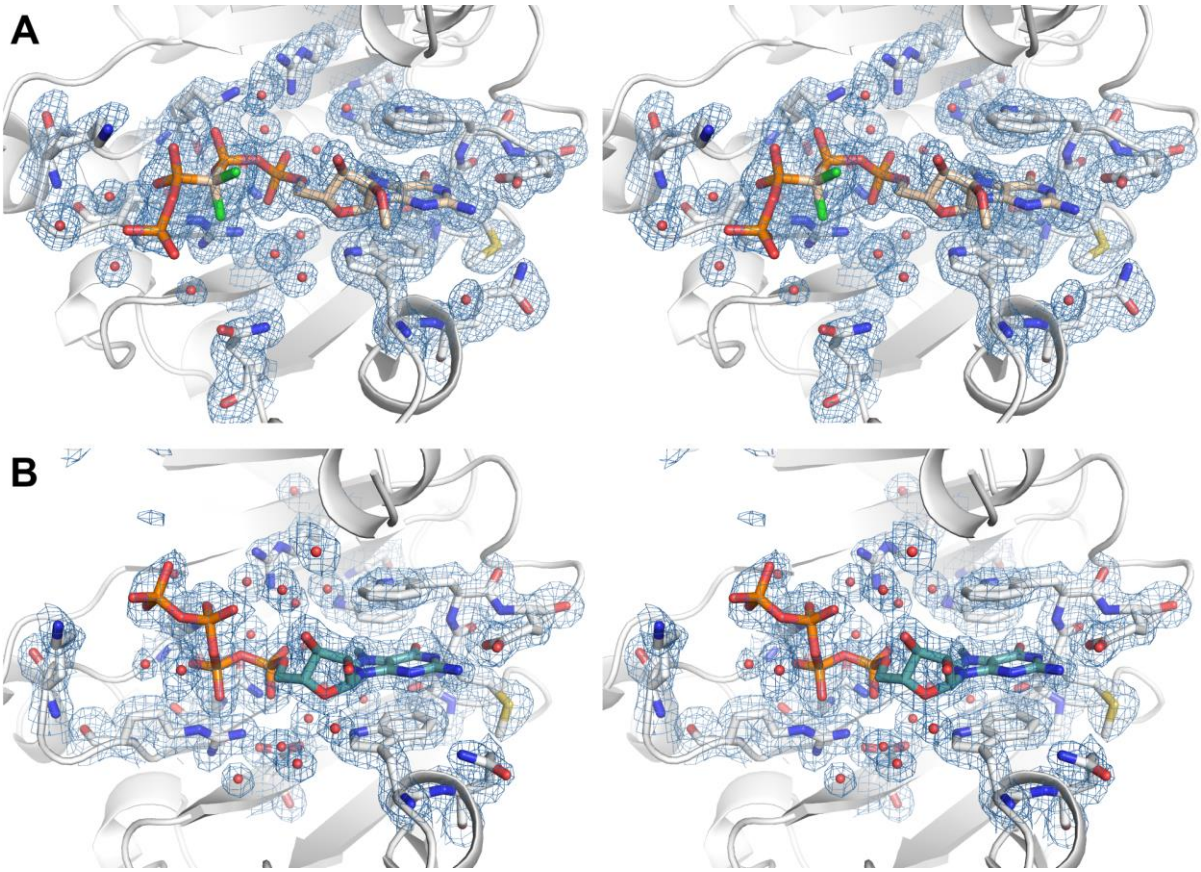
Table S4. Data collection and refinement statistics.

	eIF4E / 6d	eIF4E / 7b
PDB ID	**5J5O**	**5J5Y**
Wavelength	0.9184	0.9184
Resolution range	36.82–1.867 (1.934–1.867)	37–1.748 (1.81–1.748)
Space group	P 1	P 1
Unit cell	37.99 38.03 146.64 88.553 84.704 76.667	38.04 38.06 146.74 88.356 95.626 103.544
Total reflections	156370 (10888)	194484 (14491)
Unique reflections	60259 (5024)	72988 (6391)
Multiplicity	2.6 (2.2)	2.7 (2.3)
Completeness (%)	0.91 (0.76)	0.91 (0.78)
Mean I/sigma(I)	10.46 (2.04)	12.26 (2.46)
Wilson B-factor	19.87	20.00
R-merge	0.07081 (0.5346)	0.05243 (0.3991)
R-meas	0.08663 (0.6678)	0.06398 (0.499)
CC1/2	0.995 (0.677)	0.997 (0.825)
CC*	0.999 (0.898)	0.999 (0.951)
Reflections used in refinement	60257 (5024)	72986 (6391)
Reflections used for R-free	2234 (187)	3650 (320)
R-work	0.2209 (0.2829)	0.2364 (0.2772)
R-free	0.2637 (0.3532)	0.2860 (0.3429)
CC(work)	0.915 (0.832)	0.904 (0.824)
CC(free)	0.894 (0.733)	0.856 (0.700)
Number of non-hydrogen atoms	6401	5784
macromolecules	5880	5431
ligands	160	82

Protein residues	720	680
RMS(bonds)	0.008	0.007
RMS(angles)	1.03	0.95
Ramachandran favored (%)	96	95
Ramachandran allowed (%)	3.7	5.4
Ramachandran outliers (%)	0	0
Rotamer outliers (%)	1.9	1.6
Clashscore	6.70	5.77
Average B-factor	35.25	38.41
macromolecules	35.35	38.69
ligands	44.87	35.93
solvent	29.32	33.48
Number of TLS groups	33	33

Statistics for the highest-resolution shell are shown in parentheses.

Fig. S10 Wall-eye stereo view of eIF4E/7d cap-binding pocket with 2Fo-Fc electron density map contoured at 1.0 sigma.



A - Wall-eye stereo view of eIF4E/7b (PDB id: 5J5Y) cap-binding pocket with 2Fo-Fc electron density map contoured at 1.0 sigma; B - Wall-eye stereo view of eIF4E/6d (PDB id: 5J5O) cap-binding pocket with 2Fo-Fc electron density map contoured at 1.0 sigma.

Supplementary information – synthesis

General information

Reagents were purchased from Sigma-Aldrich and used without further purification, unless otherwise stated. Water used in the experiments was double distilled using MiliQ Milipore apparatus. Acetone was distilled over phosphorous pentoxide, triethylamine was distilled over potassium hydroxide and tetrahydrofurane was distilled over sodium. Dimethylformamide, dimethylsulfoxide and trimethylphosphate were kept over 4Å molecular sieves.

The nucleotides were purified by ion-exchange chromatography on a DEAE-Sephadex A-25 (HCO_3^- form) column. A column was loaded with the reaction mixture and washed thoroughly with water (until the eluate did not precipitate with $AgNO_3$ solution) to elute all material that does not bind to the resin. Then, the nucleotides were eluted using a linear gradient of triethylammonium hydrogen carbonate (TEAB) in deionized water. Fractions were analyzed spectrophotometrically at 260 nm and those containing the desired product were analyzed by reverse-phase HPLC and combined. After evaporation under reduced pressure with the repeated addition of ethanol to decompose TEAB, compounds were isolated as triethylammonium (TEA) salt. Yields were calculated on the basis of either sample weight or, preferably, optical density milliunits (mOD) of the product. Optical measurements for m7G mononucleotides were performed in 0.1 M phosphate buffer pH = 6 at 260 nm assuming ε_{260} = 11400 cm^{-1} M^{-1} for calculations. For guanine nucleotides and dinucleotide cap analogs measurements were conducted in 0.1 M phosphate buffer pH = 7 at 260 nm, assuming ε_{260} = 12080 cm^{-1} M^{-1} and ε_{260} = 22600 cm^{-1} M^{-1}, respectively.

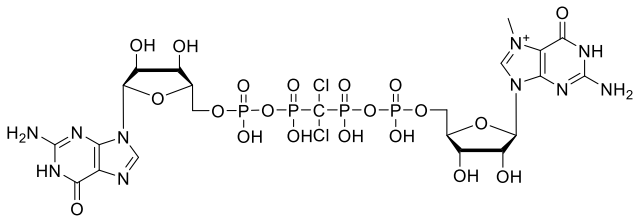
Analytical RP HPLC was performed with a Series 1200 instrument from Agilent Technologies on a Supelcosil LC-18-T HPLC column (4.6 x 250 mm, flow rate 1.3 mL min^{-1}) with a 0-25% linear gradient of methanol in 0.05 M ammonium acetate buffer (pH 5.9) for 15 min. Absorbance was monitored at 260 nm, while fluorescence was recorded at an excitation wavelength of 260 nm and an emission wavelength of 370 nm. Semi-preparative HPLC was performed on the same apparatus equipped with a Discovery Reverse-Phase Amide C-16 HPLC column (25 cm x 21.2 mm, 5 μm, flow rate 5.0 mL min^{-1}) and UV detection at 254 nm. The purity and homogeneity of each final product were confirmed by RP HPLC, high resolution mass spectrometry HRMS (ES^-) and ^1H NMR and ^{31}P NMR spectroscopy. Mass spectra were recorded with a high resolution LTQ Orbitrap Velos (Thermo Scientific). NMR spectra were recorded at 25 °C with a Varian UNITY-plus spectrometer at 399.94 MHz (^1H NMR) and 161.90 MHz (^{31}P NMR). All chemical shifts (δ) are given in ppm and coupling constants (J) are given in Hz. ^1H NMR chemical shifts were calibrated to sodium 3-trimethylsilyl-[2,2,3,3-D_4]-propionate (TSP) in D2O as an internal standard. ^{31}P NMR chemical shifts were reported to 20% phosphorus acid in D_2O as an external standard. The raw NMR files were processed using ACD/Labs 12.0 Software.

Synthesis of previously described compounds

Methylenebisphosphonate containing analogues: m^7GppCH_2ppG (**6a**)[1] and $m_2^{7,2'\text{-}O}GppCH_2ppG$ (**7a**)[1], imidazolide derivatives: $m^7Gmp\text{-}Im$ (**1**)[3], Gmp-Im (**3**)[4] and $m_2^{7,2'\text{-}O}Gmp\text{-}Im$ (**5**)[4] and analogues unmodified in the polyphosphate bridge: $m^7GppppG$ (**6d**)[5], $m_2^{7,2'\text{-}O}GppppG$ (**7d**)[4], $m_2^{7,3'\text{-}O}GppppG$[4], m^7GpppG[5] and m^7Gppp (**2d**) were obtained as previously described.

Synthesis of $m^7GppCCl_2ppG$

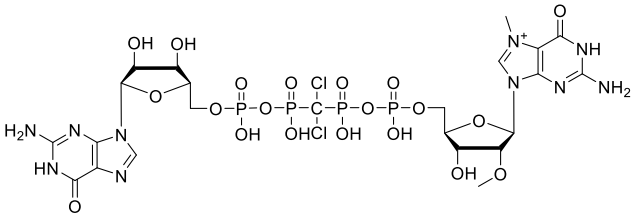
P1-(7-methylguanosin-5´-yl) P4-guanosin-5´-yl 2,3-dichloromethylenetetraphosphate



m^7Gmp-Im (108 mg, 0.24 mmol) and $GppCCl_2p$ (TEA salt, 85 mg, 0.095 mmol) were mixed in anhydrous DMF (4 ml) followed by addition of anhydrous $ZnCl_2$ (103 mg, 0.76 mmol). After 4 h reaction was completed and quenched by addition of solution of EDTA (283 mg, 0.76 mmol) and $NaHCO_3$ (142 mg, 1.69 mmol) in water. Product was purified employing ion-exchange chromatography (Sephadex resin) and obtained as glassy solid (TEA salt, 1188 opt. u., 0.057 mmol, 60%). Further purification was performed using preparative HPLC yielding in 35.5 mg of final compound (NH_4^+ salt, 0.035 mmol, 37%). **^{1}H NMR** (400 MHz, D_2O): δ 9.20 (1 H, s, $H8_{m7G}$), 8.05 (1 H, s, $H8_G$), 5.98 (1H, d, $J_{1'-2'}$ = 3.3 Hz, $H1'_{m7G}$), 5.84 (1H, d, $J_{1'-2'}$ = 6.2 Hz, $H1'_G$), 4.73 (1H, dd, $J_{1'-2'}$ = 6.2 Hz, $J_{2'-3'}$ = 5.4 Hz, $H2'_G$), 4.64 (1H, dd, $J_{1'-2'}$ = 3.3 Hz, $J_{2'-3'}$ = 5.1 Hz, $H2'_{m7G}$), 4.54 (1H, m, $H3'_{m7G}$), 4.50 (1H, dd, $J_{2'-3'}$ = 5.4 Hz, $J_{3'-4'}$ = 4.4 Hz, $H3'_G$), 4.41-4.25 (6H, m; $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.08 (3H, s, CH_3); **^{31}P NMR** (162 MHz, D_2O) δ -10.81 (2P; Pα,δ), -1.41 (2P; Pβ,γ). **HRMS** (ESI^-) calc. for $C_{22}H_{29}Cl_2N_{10}O_{20}P_4^-$ requires 946.9893, found 946.9901.

Synthesis of $m_2^{7,2'\text{-}O}GppCCl_2ppG$

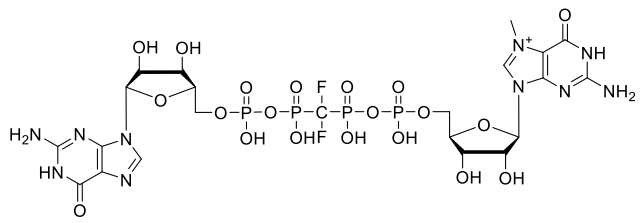
P1-(7, 2'-O-dimethylguanosin-5´-yl) P4-guanosin-5´-yl 2,3-dichloromethylenetetraphosphate



$m_2^{7,2'\text{-}O}$Gmp-Im (90 mg, 0.19 mmol) and $GppCCl_2p$ (TEA salt, 57 mg, 0.064 mmol) were mixed in anhydrous DMF (3 ml) followed by addition of anhydrous $ZnCl_2$ (70 mg, 0.51 mmol). After 4 h reaction was completed and quenched by addition of solution of EDTA (190 mg, 0.51 mmol) and $NaHCO_3$ (95 mg, 1.13 mmol) in water. Product was purified employing ion-exchange chromatography (Sephadex resin) and obtained as glassy solid (TEA salt, 900 opt. u., 0.043 mmol, 67%). Final compound was changed into sodium salt on Dowex (Na^+ form) yielding 36.7 mg of final compound (0.035 mmol, 55%). **^{1}H NMR** (400 MHz, D_2O): δ 9.20 (1 H, s, $H8_{m7G}$), 8.76 (1 H, s, $H8_G$), 6.07 (1H, d, $J_{1'-2'}$ = 3.1 Hz, $H1'_{m7G}$), 5.96 (1H, d, $J_{1'-2'}$ = 4.6 Hz, $H1'_G$), 4.68 (1H, t, $J_{1'-2'/2'-3'}$ = 4.8 Hz, $H2'_G$), 4.60 (1H, t, $J_{2'-3'/3'-4'}$ = 5.3 Hz, $H3'_{m7G}$), 4.54 (1H, t, $J_{2'-3'/3'-4'}$ = 4.4 Hz, $H3'_G$), 4.40-4.24 (6H, m; $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.10 (3H, s, CH_3), 3.58 (3H, s OCH_3); **^{31}P NMR** (162 MHz, D_2O) δ -10.78 (2P; Pα,δ), -1.35 (2P; Pβ,γ). **HRMS** (ESI^-) calc. for $C_{23}H_{31}Cl_2N_{10}O_{20}P_4^-$ requires 961.0049, found 961.0056.

Synthesis of m^7GppCF_2ppG

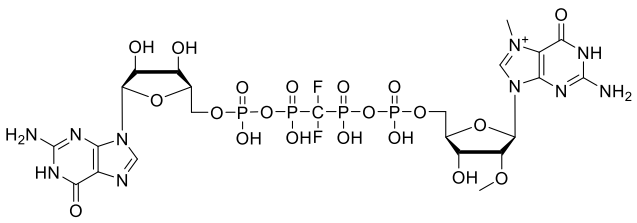
P1-(7-methylguanosin-5´-yl) P4-guanosin-5´-yl 2,3-difluoromethylenetetraphosphate



m^7Gmp-Im (50 mg, 0.132 mmol) and $GppCF_2p$ (ammonium salt, 55 mg, 0.088 mmol) were mixed in anhydrous DMF (2 ml) followed by addition of anhydrous $ZnCl_2$ (96 mg, 0.70 mmol). After 4 h reaction was completed and quenched by addition of solution of EDTA (260 mg, 0.70 mmol) and $NaHCO_3$ (130 mg, 1.56 mmol) in water. Product was purified employing ion-exchange chromatography (Sephadex resin) and obtained as glassy solid (TEA salt, 920 opt. u., 0.044 mmol, 50%). Further purification was performed using preparative HPLC yielding in 17.7 mg of final compound (NH_4^+ salt, 0.018 mmol, 20%). Final compound was changed into sodium salt on Dowex (Na^+ form) yielding 17.9 mg of final compound (0.018 mmol, 20%). **^{1}H NMR** (400 MHz, D_2O): δ 9.12 (1 H, s, $H8_{m7G}$), 8.04 (1 H, s, $H8_G$), 5.98 (1H, d, $J_{1'-2'}$ = 3.7 Hz, $H1'_{m7G}$), 5.85 (1H, d, $J_{1'-2'}$ = 6.5 Hz, $H1'_G$), 4.74 (1H, t, $J_{1'-2'/2'-3'}$ = 5.7 Hz, $H2'_G$), 4.65 (1H, t, $J_{1'-2'/2'-3'}$ = 4.4 Hz, $H2'_{m7G}$), 4.52 (1H, dd, $J_{2'-3'}$ = 5.0 Hz, $J_{4'-3'}$ = 3.0 Hz, $H3'_{m7G}$), 4.49 (1H, t, $J_{2'-3'/4'-3'}$ = 4.9 Hz, $H3'_G$), 4.19-4.43 (6H, m; $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.08 (3H, s, CH_3); **^{31}P NMR** (162 MHz, D_2O) δ -11.08 (2P; Pα,δ), -6.37 (2P; Pβ,γ). **HRMS** (ESI^-) calc. for $C_{22}H_{29}F_2N_{10}O_{20}P_4^-$ requires 915.0484, found 915.0501.

Synthesis of $m_2^{7,2'\text{-}O}GppCF_2ppG$

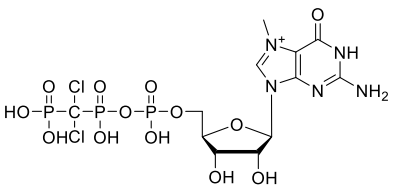
P1-(7,2'-O-dimethylguanosin-5´-yl) P4-guanosin-5´-yl 2,3-difluoromethylenetetraphosphate



$m^{7,2'\text{-}O}Gmp$-Im (45 mg, 0.096 mmol) and $GppCF_2p$ (ammonium salt, 40 mg, 0.064 mmol) were mixed in anhydrous DMF (1 ml) followed by addition of anhydrous $ZnCl_2$ (32 mg, 0.24 mmol). After 4 h reaction was completed and quenched by addition of solution of EDTA (90 mg, 0.24 mmol) and $NaHCO_3$ (42 mg, 0.50 mmol) in water. Product was purified employing ion-exchange chromatography (Sephadex resin) and obtained as glassy solid (TEA salt, 1010 opt. u., 0.048 mmol, 55%). Further purification was performed using preparative HPLC yielding in 20.0 mg of final compound (NH_4^+ salt, 0.020 mmol, 37%). Final compound was changed into sodium salt on Dowex (Na^+ form) yielding 20.5 mg of final compound (0.021 mmol, 33%). **^{1}H NMR** (400 MHz, D_2O): δ 8.02 (1 H, s, $H8_G$), 6.01 (1H, br.s., $H1'_{m7G}$), 5.82 (1H, d, $J_{1'-2'}$ = 6.7 Hz, $H1'_G$), 4.73 (1H, t, $J_{1'-2'/2'-3'}$ = 5.6 Hz, $H2'_G$), 4.56 (1H, t, $J_{2'-3'/3'-4'}$ = 5.4 Hz, $H3'_{m7G}$), 4.49-4.53 (1H, m, $H3'_G$), 4.20-4.43 (6H, m; $H4'_{m7G}$, $H4'_G$, $H5'_{m7G}$, $H5'_G$, $H5''_{m7G}$, $H5''_G$), 4.09 (3H, s, CH_3), 3.59 (3H, s OCH_3); **^{31}P NMR** (162 MHz, D_2O) δ -11.11 (2P; Pα,δ), -6.35 (2P; Pβ,γ). **HRMS** (ESI^-) calc. for $C_{23}H_{31}F_2N_{10}O_{20}P_4^-$ requires 929.0640, found 929.0656.

Synthesis of $m^7GppCCl_2p$

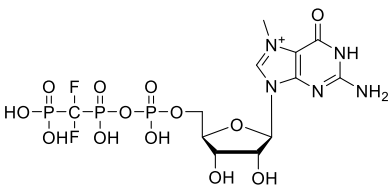
P1-(7-methylguanosin-5'-yl) 2,3-(dichloromethylene)triphosphate



To a suspension of dichlorobisphosphonate triethylammonium salt (400 mg, 1.20 mmol) in DMF (4 ml) anhydrous zinc chloride (164 mg, 1.21 mmol) was added, and the mixture was shaken until reagents dissolved. Then 7-methylguanosine imidazolide (1700 opt. u., 0.15 mmol) was added, followed by addition of another portion of zinc chloride (164 mg, 1.21 mmol). The reaction was quenched after 4 hours by addition of water solution of EDTA (897 mg, 2.41 mmol) and $NaHCO_3$ (449 mg, 5.34 mmol). Product was purified employing ion-exchange chromatography on Sephadex resin in linear gradient from 0 to 1.1 M TEAB. Collected fractions were evaporated with several additions of ethanol yielding $m^7GppCCl_2p$ as treithylammonium salt (1020 opt.u., 0.089 mmol, 60%). Further purification was done by preparative HPLC. Collected fractions were freeze-dried severeal times until mass of the sample remained constant. Product was obtained as ammonium salt (26.6 mg, 0.041 mmol, 27%). **^{1}H NMR** (400 MHz, D_2O): δ 6.07 (1H, d, $J_{1'-2'}$ = 3.4 Hz, H1'), 4.70 (1H, dd, $J_{1'-2'}$ = 3.4 Hz, $J_{2'-3'}$ = 4.6 Hz, H2'), 4.58 (1H, dd, $J_{2'-3'}$ = 4.6 Hz, $J_{3'-4'}$ = 5.7 Hz, H3'), 4.43-4.30 (3H, m, H4', H5', H5''), 4.14 (3H, s, CH_3); **^{31}P NMR** (162 MHz, D_2O) δ 7.90 (1P, d, *J* = 17.8Hz, Pγ), 0.88 (1P, dd, *J* = 17.5Hz, *J* = 29.9 Hz, Pβ), -10.58 (1P, d, *J* = 30.3 Hz, Pα). **HRMS** (ESI^-) calc. for $C_{12}H_{17}Cl_2N_5O_{13}P_3^-$ requires 601.9418, found 601.9425.

Synthesis of m^7GppCF_2p

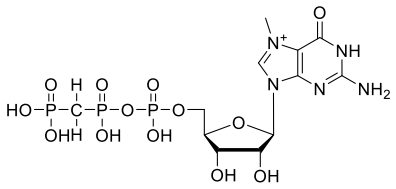
P1-(7-methylguanosin-5'-yl) 2,3-(difluoromethylene)triphosphate



To a suspension of difluorobisphosphonate triethylammonium salt (327 mg, 0.79 mmol) in DMF (4 ml) anhydrous zinc chloride (150 mg, 1.10 mmol) was added, and the mixture was shaken until reagents dissolved. Then 7-methylguanosine imidazolide (1230 opt. u., 0.11 mmol) was added, followed by addition of another portion of zinc chloride (150 mg, 1.10 mmol). The reaction was quenched after 4 hours by addition of water solution of EDTA (821 mg, 2.21 mmol) and $NaHCO_3$ (410 mg, 5.34 mmol). Product was purified employing ion-exchange chromatography on Sephadex resin in linear gradient from 0 to 1.1 M TEAB. Collected fractions were evaporated with several additions of ethanol yielding m^7GppCF_2p as treithylammonium salt (1032 opt.u., 0.091 mmol, 82%). Further purification was done by preparative HPLC. Collected fractions were freeze-dried severeal times until mass of the sample remained constant. Product was obtained as ammonium salt (31 mg, 0.049 mmol, 44%). **^{1}H NMR** (400 MHz, D_2O) δ ppm 9.22 (1H, s, H8), 6.08 (1H, d, $J_{1'-2'}$ = 3.7 Hz, H1'), 4.71 (1H, dd, $J_{1'-2'}$ = 3.7 Hz, $J_{2'-3'}$ = 4.7 Hz, H2'), 4.58 (1H, dd, $J_{2'-3'}$ = 4.7 Hz, $J_{3'-4'}$ = 5.5 Hz, H3'), 4.39 - 4.43 (1H, m, H4'), 4.35 (1H, m, H5'), 4.31 (1H, m, H5''), 4.14 (3H, s, CH_3). **^{31}P NMR** (162 MHz, D_2O) δ 3.49 (1P, td, *J* = 75 Hz, J = 57 Hz, Pγ), -3.51 (1P, m, Pβ), -10.90 (1P, d, *J* = 31.0 Hz, Pα). **HRMS** (ESI^-) calc. for $C_{12}H_{17}F_2N_5O_{13}P_3^-$ requires 570.0009, found 570.0014.

Synthesis of m^7GppCH_2p

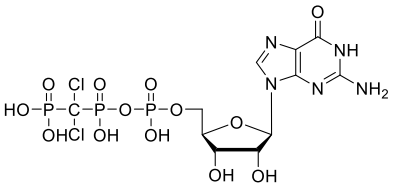
P1-(7-methylguanosin-5'-yl) 2,3-methylenetriphosphate



To a suspension of bisphosphonate triethylammonium salt (359 mg, 0.95 mmol) in DMF (4 ml) anhydrous zinc chloride (240 mg, 1.76 mmol) was added, and the mixture was shaken until reagents dissolved. Then 7-methylguanosine imidazolide (1248 opt. u., 0.11 mmol) was added, followed by addition of another portion of zinc chloride (240 mg, 1.76 mmol). The reaction was quenched after 4 hours by addition of water solution of EDTA (1.313 g, 3.53 mmol) and $NaHCO_3$ (657 mg, 7.82 mmol). Product was purified employing ion-exchange chromatography on Sephadex resin in linear gradient from 0 to 1.1 M TEAB. Collected fractions were evaporated with several additions of ethanol yielding m^7GppCH_2p as treithylammonium salt (673 opt.u., 0.059 mmol, 54%). Product was changed into sodium salt on Dowex resin (Na^+ form). **^{1}H NMR** (400 MHz, D_2O) δ ppm 5.96 (1H, d, $J_{1'-2'}$ = 3.6 Hz, H1'), 4.56 (1H, dd, $J_{1'-2'}$ = 3.7 Hz, $J_{2'-3'}$ = 4.9 Hz, H2'), 4.42 (1H, t, J = 5.2 Hz, H3'), 4.29 (1H, dq, $J_{3'-4'}$ = 5.1 Hz, $J_{4'-5}$ = 2.6 Hz, H4'), 4.23 (1H, m, H5'), 4.15 (1H, m, H5''), 4.01 (s, 3H), 2.21 (dd, J = 20.8, J = 19.9 Hz, 2H). **^{31}P NMR** (162 MHz, D_2O) δ 15.03 (1P, d, J = 8.8 Hz, Pγ), 10.21 (1P, dd, J = 26.5, J = 8.9, Pβ), -10.16 (1P, d, J = 26.4 Hz, Pα). **HRMS** (ESI^-) calc. for $C_{12}H_{19}N_5O_{13}P_3^-$ requires 534.0192, found 534.0195.

Synthesis of $GppCCl_2p$

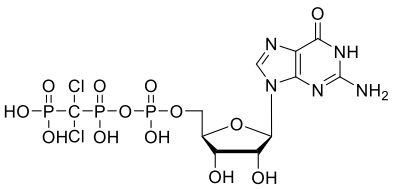
P1-guanosin-5'-yl-2,3-(dichloromethylene)triphosphate



To a suspension of dichlorobisphosphonate triethylammonium salt (720 mg, 1.61 mmol) in DMF (5 ml) anhydrous zinc chloride (218 mg, 1.61 mmol) was added, and the mixture was shaken until reagents dissolved. Then guanosine monophosphate imidazolide (200mg, 0.46 mmol) was added followed by addition of another portion of zinc chloride (218 mg, 1.61 mmol). The reaction was quenched after 4 hours by addition of water solution of EDTA (1120 mg, 3.2 mmol) and $NaHCO_3$ (590 mg, 7.04 mmol). Product was purified employing ion-exchange chromatography on Sephadex resin in linear gradient from 0 to 1.2 M TEAB. Collected fractions were evaporated with several additions of ethanol yielding $GppCCl_2p$ as treithylammonium salt (370 mg, 0.41 mmol, 89%). **^{1}H NMR** (400 MHz, D_2O): δ 8.12 (1H, s, $H8_G$), 5.93 (1H, d, $J_{1'-2'}$ = 6.5 Hz, H1'), 4.85 (1H, t, $J_{1'-2'/2'-3'}$ = 5.7 Hz, H2'), 4.60 (1H, dd, $J_{2'-3'}$ = 5.2 Hz, $J_{3'-4'}$ = 3.2 Hz, H3'), 4.36 (1H, m, H4') 4.33-4.21 (2H, m, H5', H5''), 3.21 (q, J = 7.5 Hz, CH_2CH_3), 1.29 (t, J = 7.3 Hz, CH_2CH_3); **^{31}P NMR** (162 MHz, D_2O) δ 7.71 (1P, d, J = 17.8 Hz, Pγ), -0.65 (1P, dd, J = 19.4 Hz, J = 30.1 Hz, Pβ), -10.61 (1P, d, J = 29.8 Hz, Pα). **HRMS** (ESI^-) calc. for $C_{11}H_{15}Cl_2N_5O_{13}P_3^-$ requires 587.9262, found 587.9267.

Synthesis of GppCCl$_2$p

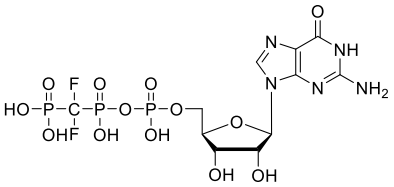
P1-guanosin-5'-yl-2,3-(dichloromethylene)triphosphate



To a suspension of dichlorobisphosphonate triethylammonium salt (720 mg, 1.61 mmol) in DMF (5 ml) anhydrous zinc chloride (218 mg, 1.61 mmol) was added, and the mixture was shaken until reagents dissolved. Then guanosine monophosphate imidazolide (200mg, 0.46 mmol) was added followed by addition of another portion of zinc chloride (218 mg, 1.61 mmol). The reaction was quenched after 4 hours by addition of water solution of EDTA (1120 mg, 3.2 mmol) and $NaHCO_3$ (590 mg, 7.04 mmol). Product was purified employing ion-exchange chromatography on Sephadex resin in linear gradient from 0 to 1.2 M TEAB. Collected fractions were evaporated with several additions of ethanol yielding GppCCl$_2$p as treithylammonium salt (370 mg, 0.41 mmol, 89%). **^{1}H NMR** (400 MHz, D_2O): δ 8.12 (1H, s, H8$_G$), 5.93 (1H, d, $J_{1'\text{-}2'}$ = 6.5 Hz, H1'), 4.85 (1H, t, $J_{1'\text{-}2'/2'\text{-}3'}$ = 5.7 Hz, H2'), 4.60 (1H, dd, $J_{2'\text{-}3'}$ = 5.2 Hz, $J_{3'\text{-}4'}$ = 3.2 Hz, H3'), 4.36 (1H, m, H4') 4.33-4.21 (2H, m, H5', H5''), 3.21 (q, J = 7.5 Hz, CH_2CH$_3$), 1.29 (t, J = 7.3 Hz, CH$_2$CH_3); **^{31}P NMR** (162 MHz, D_2O) δ 7.71 (1P, d, J = 17.8 Hz, Pγ), -0.65 (1P, dd, J = 19.4 Hz, J = 30.1 Hz, Pβ), -10.61 (1P, d, J = 29.8 Hz, Pα). **HRMS** (ESI$^-$) calc. for $C_{11}H_{15}Cl_2N_5O_{13}P_3^-$ requires 587.9262, found 587.9267.

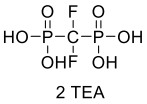
Synthesis of GppCF$_2$p

P1-guanosin-5'-yl-2,3-(difluoromethylene)triphosphate



To a suspension of dichlorobisphosphonate triethylammonium salt (700 mg, 1.70 mmol) in DMF (5 ml) anhydrous zinc chloride (218 mg, 1.61 mmol) was added, and the mixture was shaken until reagents dissolved. Then guanosine monophosphate imidazolide (200mg, 0.46 mmol) was added followed by addition of another portion of zinc chloride (218 mg, 1.61 mmol). The reaction was quenched after 4 hours by addition of water solution of EDTA (1120 mg, 3.2 mmol) and $NaHCO_3$ (590 mg, 7.04 mmol). Product was purified employing ion-exchange chromatography on Sephadex resin in linear gradient from 0 to 1.2 M TEAB. Collected fractions were evaporated with several additions of ethanol yielding GppCF$_2$p as treithylammonium salt (224mg, 0.26 mmol, 57%). **^{1}H NMR** (400 MHz, D_2O): δ 8.30 (1H, s, H8$_G$), 5.86 (1H, d, $J_{1'\text{-}2'}$ = 5.5 Hz, H1'), 4.62 (1H, t, $J_{1'\text{-}2'/2'\text{-}3'}$ = 5.2 Hz, H2'), 4.45 (1H, dd, $J_{2'\text{-}3'}$/$_{3'\text{-}4'}$ = 4.2 Hz, H3'), 4.28 (1H, m, H4') 4.16 (2H, m, H5', H5''), 3.10 (q, J = 7.3 Hz, CH_2CH$_3$), 1.18 (t, J = 7.3 Hz, CH$_2$CH_3); **^{31}P NMR** (162 MHz, D_2O) δ 7.71 (1P, td, J = 77.9×2, J = 58.8 Hz, Pγ), -4.08 (1P, m, Pβ), -10.63 (1P, d, J = 31.3 Hz, Pα). **HRMS** (ESI$^-$) calc. for $C_{11}H_{15}F_2N_5O_{13}P_3^-$ requires 555.9853, found 555.98538.

Synthesis of methylenedifluorobisphosphonate

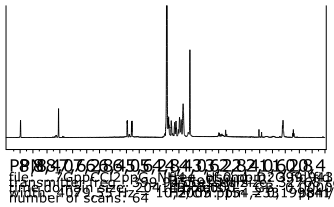


Tetraisopropyl methylenebisphosphonate (2.6 mL, 8.1 mmol) was placed in the oven dried two-neck roundbottom flask fitted with reflux condenser and flushed with argon. To the flask 20 mL of NaHMDS (1 M solution in THF, 20 mmol) was added and mixture was stirred for 5 min. To the resultant mixture solution of N-Fluorobenzenesulfonimide (NFSi) in dry THF (9.58 g of NFSi reagent dissolved in 30 mL of dry THF, 30.4 mmol) was added in the increments of 6 mL. Addition of each increment of NFSi solution was followed by addition of 6 mL of 1M THF solution of NaHMDS (to the total volume of 24 mL of NaHMDS solution). During additions the formation of creamy brown precipitate was observed. Reaction was stirred for additional hour and the precipitate was filtered off and washed with hexane. The filtrate was concentrated *in vacuo* to yield brown oil which dissolved in dichloromethylene enad washed with 1M aqueous solution of sodium bicarbonate. Organic layer was dried over magnesium sulphate, filtered, concentrated *in vacuo* and subjected to column chromatography on silica (chloroform/ ethyl acetate, 0-50%). Product was eluted with 15% ethyl acetate, followed by monofluorination product and unreacted substrate. The tetraisopropyl difluorobisphosphonate was obtained as a pale yellow oil (1.32 g, 3.5 mmol, 43%).

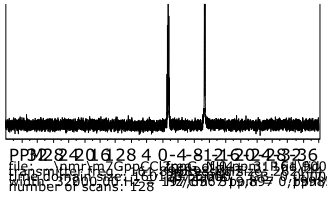
The obtained tetraisopropyl difluorobisphosphonate (1.32 g, 3.5 mmol) was dissolved in dichloromethylene (5 mL) and then transferred to the flask fitted with reflux condenser with tube filled with calcium chloride. To resulting solution TMSBr was added (2.3 mL, 17.4 mmol) and mixture was refluxed for 16 h. Afterwards flask was cooled down to the room temperature and 2.5 mL of methanol was added dropwise. Resultant brown solution was evaporated with two portions (10 mL) of methanol and then treated with 25 mL of water. Mixture was extracted with ethyl acetate until aqueous solution become colourless. The trimethylamine was added (0.98 mL, 7 mmol) to the aqueous phase and resulting mixture was evaporated *in vacuo* to yield pale brown glassy solid.

^{19}F NMR (376 MHz, D_2O): δ -121.42 (2F, t, *J* = 83.7 Hz); **^{31}P NMR** (162 MHz, D_2O) δ 3.44 (2P, t, *J* = 83.8 Hz). **HRMS** (ESI^-) calc. for $CH_3F_2O_6P_2^-$ requires 210.9378, found 210.9371.

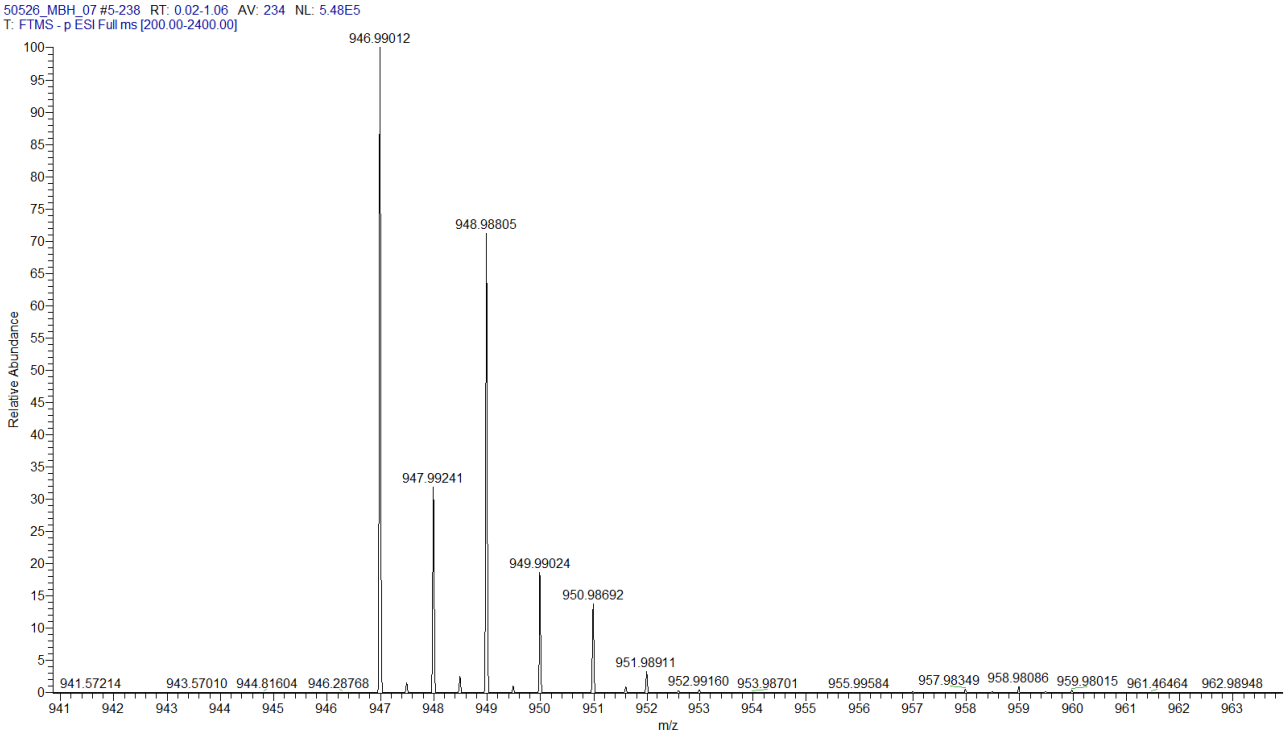
^{1}H NMR spectrum of m^{7}GppCCl$_{2}$ppG



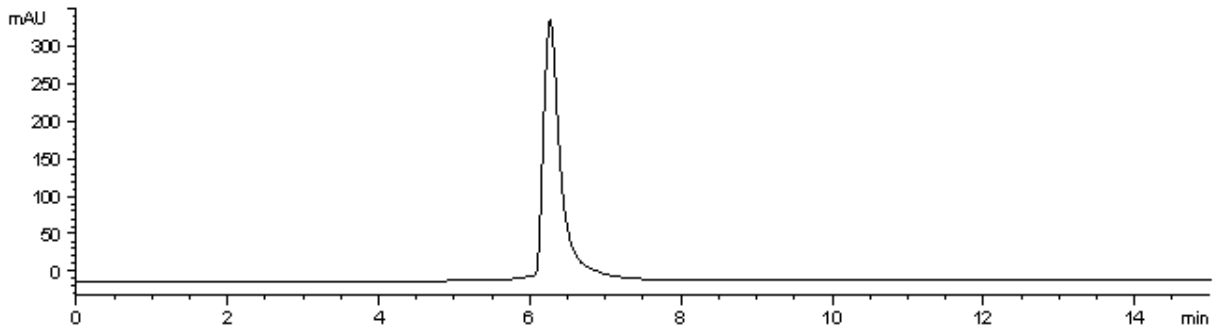
^{31}P NMR spectrum of m^{7}GppCCl$_{2}$ppG



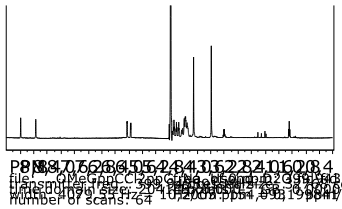
HRMS spectrum of $m^7GppCCl_2ppG$



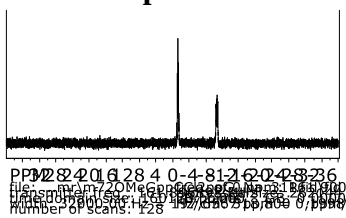
HPLC profile of $m^7GppCCl_2ppG$



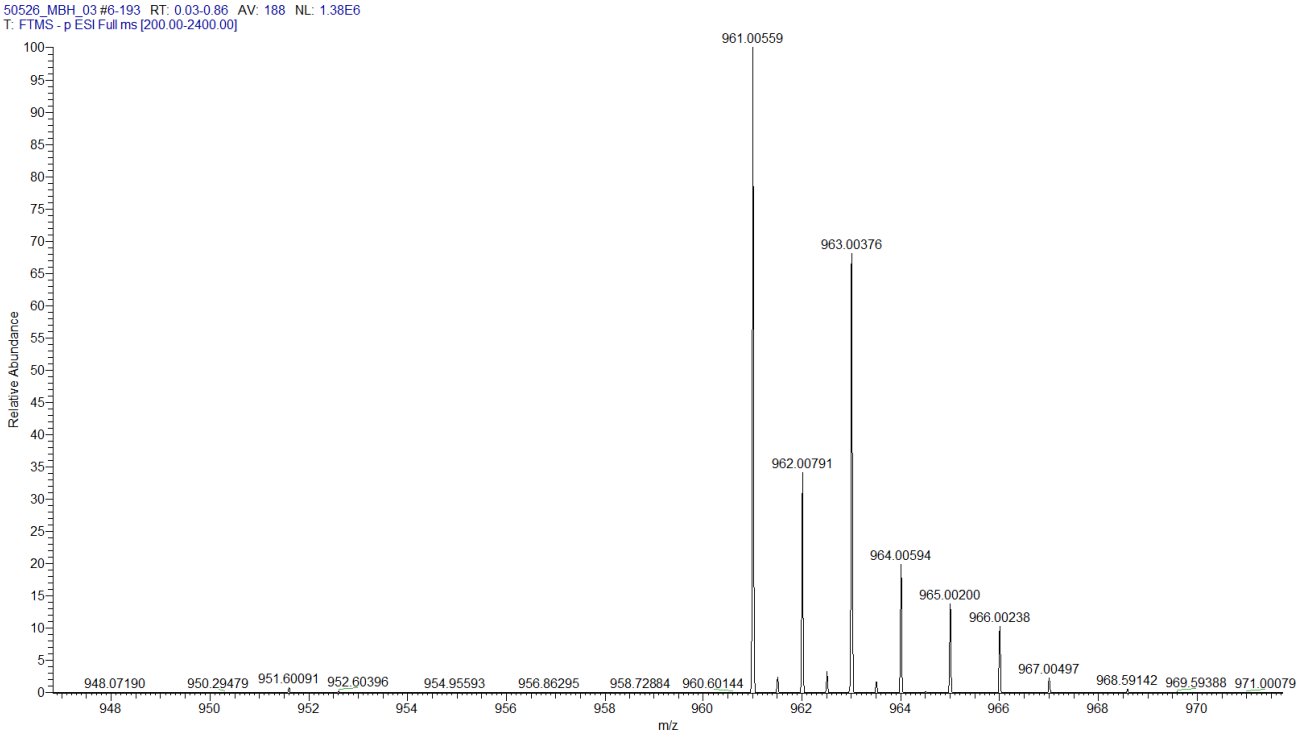
^{1}H NMR spectrum of $m_2^{7,2'\text{-}O}GppCCl_2ppG$



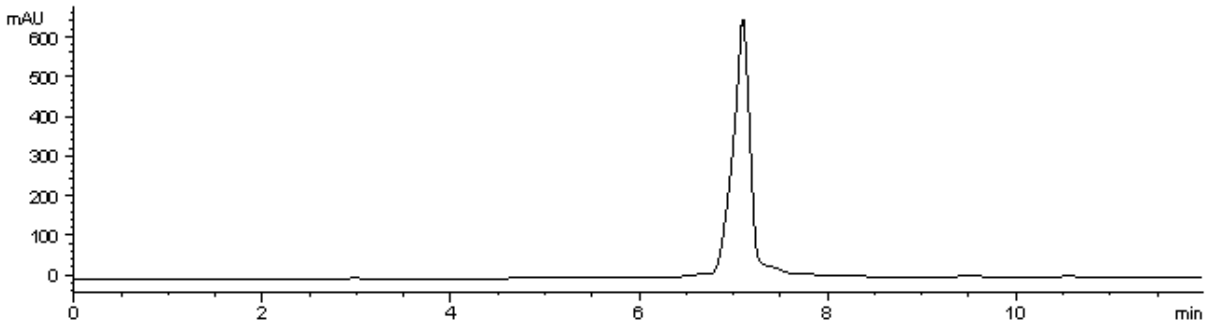
^{31}P NMR spectrum of $m_2^{7,2'\text{-}O}GppCCl_2ppG$



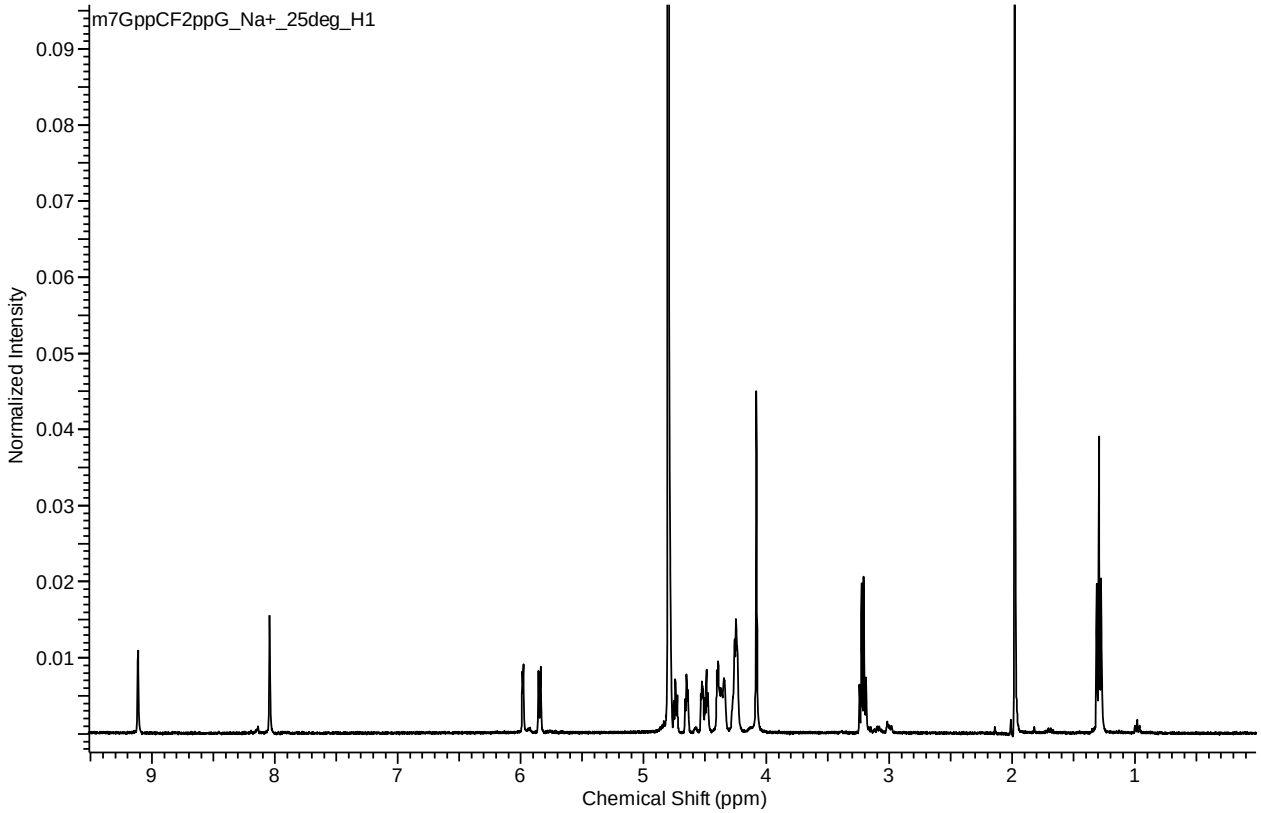
HRMS spectrum of $m_2^{7,2'\text{-}O}GppCCl_2ppG$



HPLC profile of $m_2^{7,2'\text{-}O}GppCCl_2ppG$



^{1}H NMR spectrum of m^{7}GppCF$_{2}$ppG



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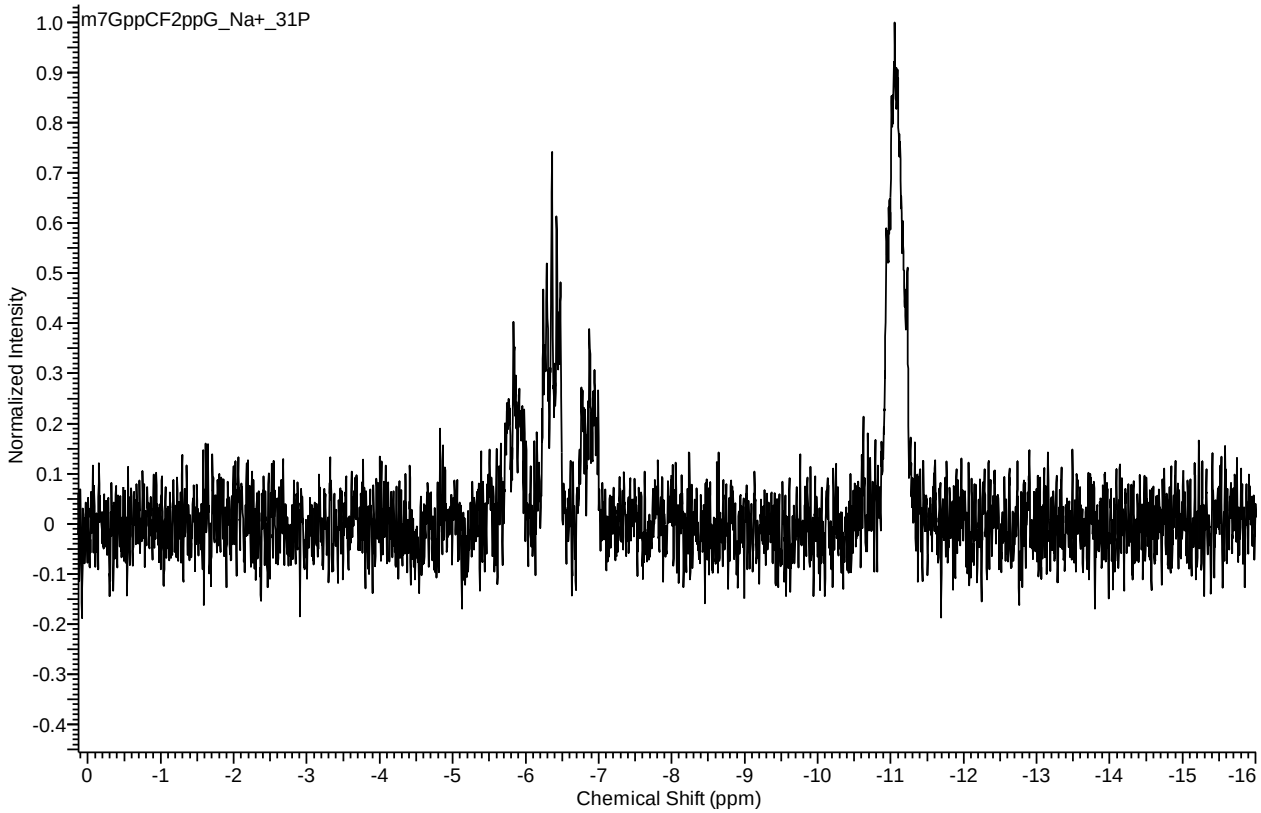

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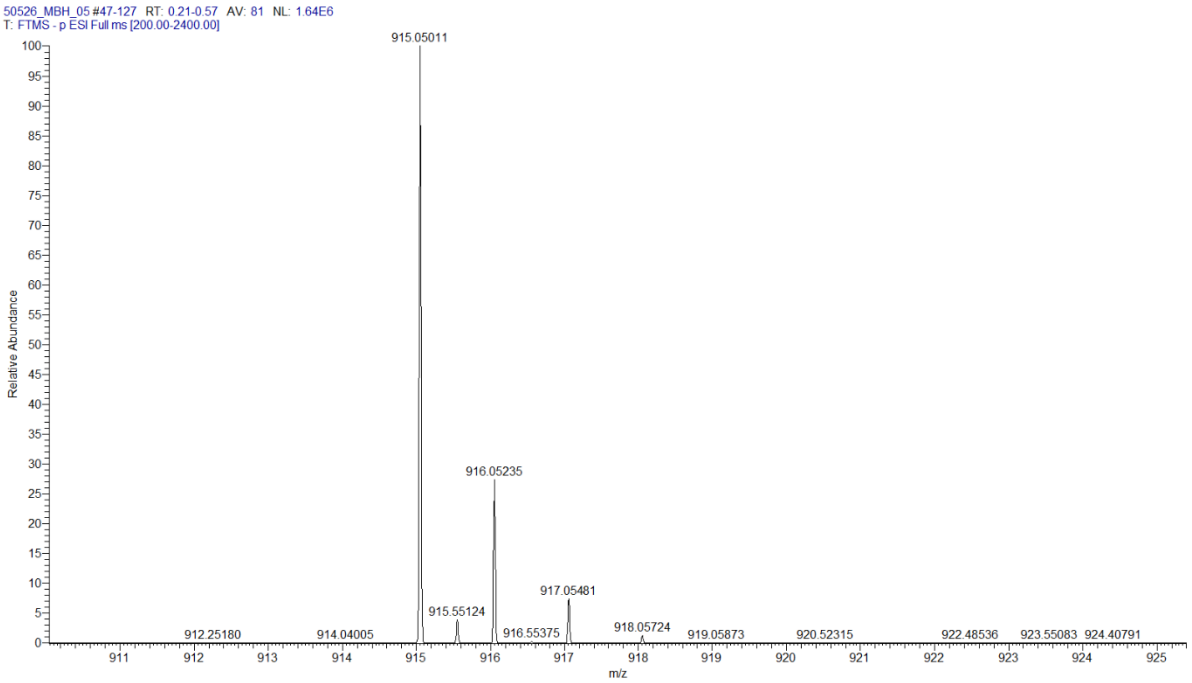
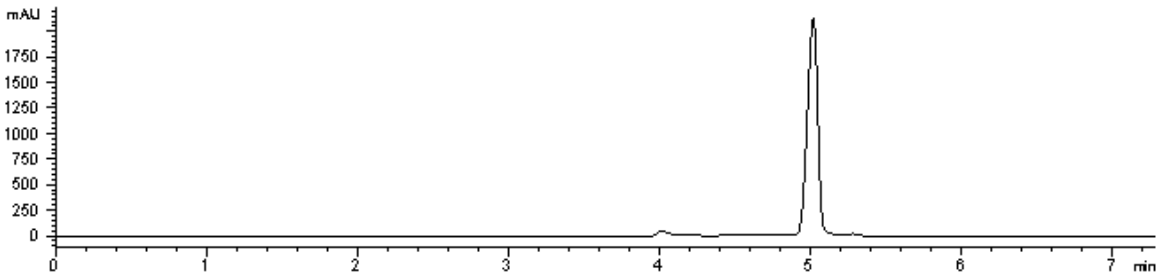


HPLC profile of m^7GppCF_2ppG



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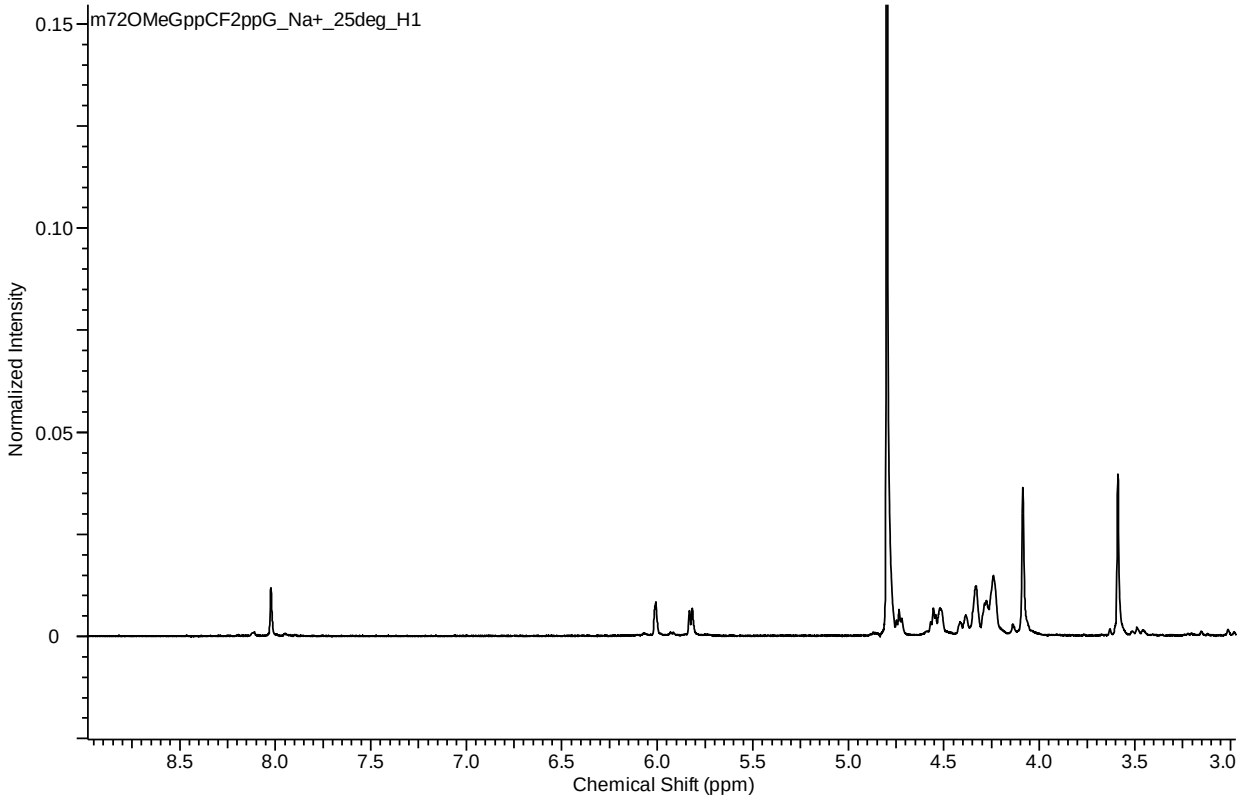
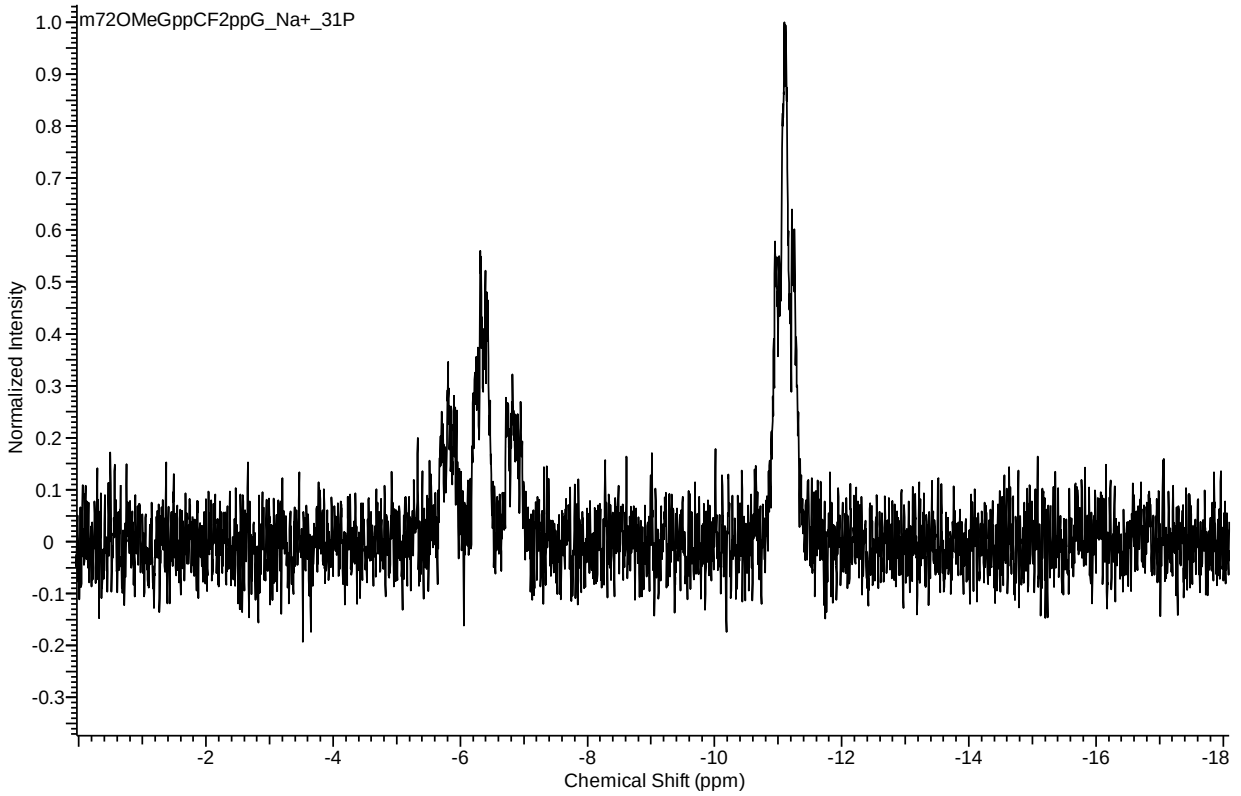
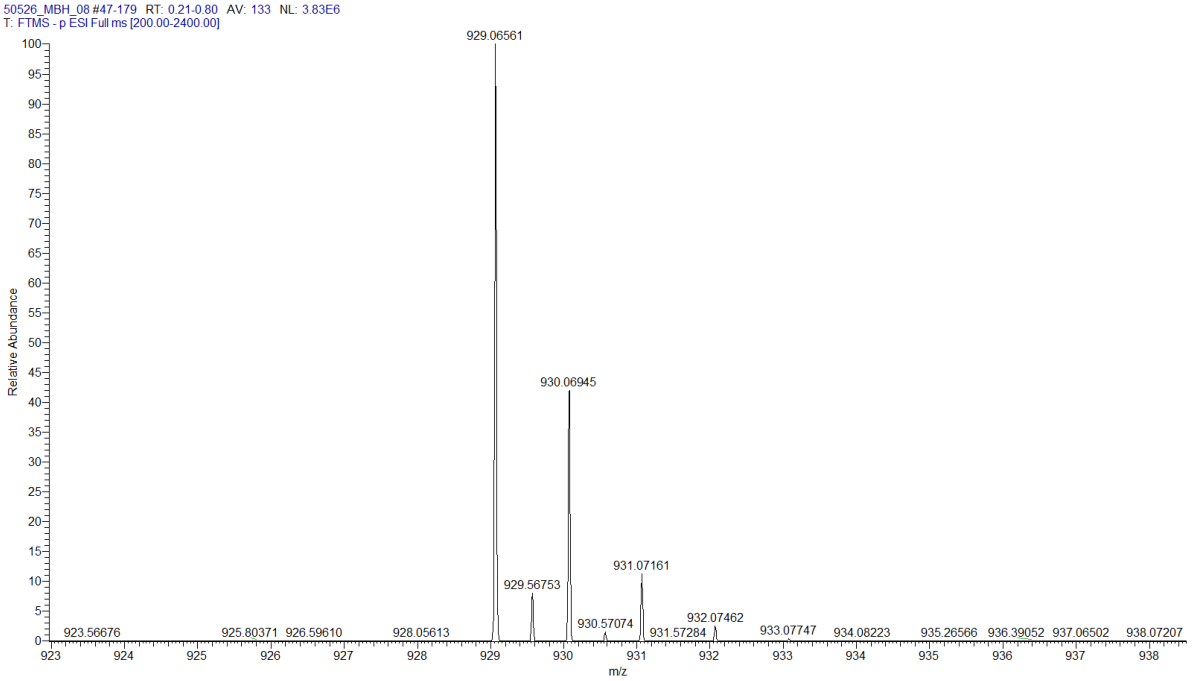


^{31}P NMR spectrum of $m_2^{7,2'\text{-}O}GppCF_2ppG$



HRMS spectrum of $m_2^{7,2'\text{-}O}GppCF_2ppG$



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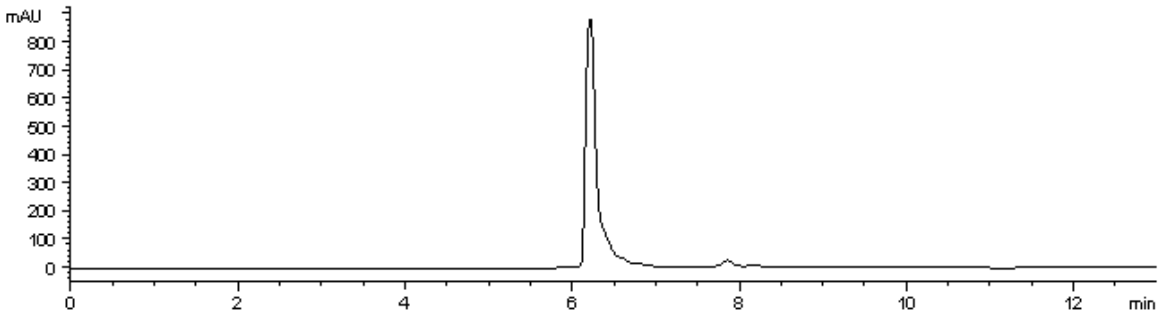
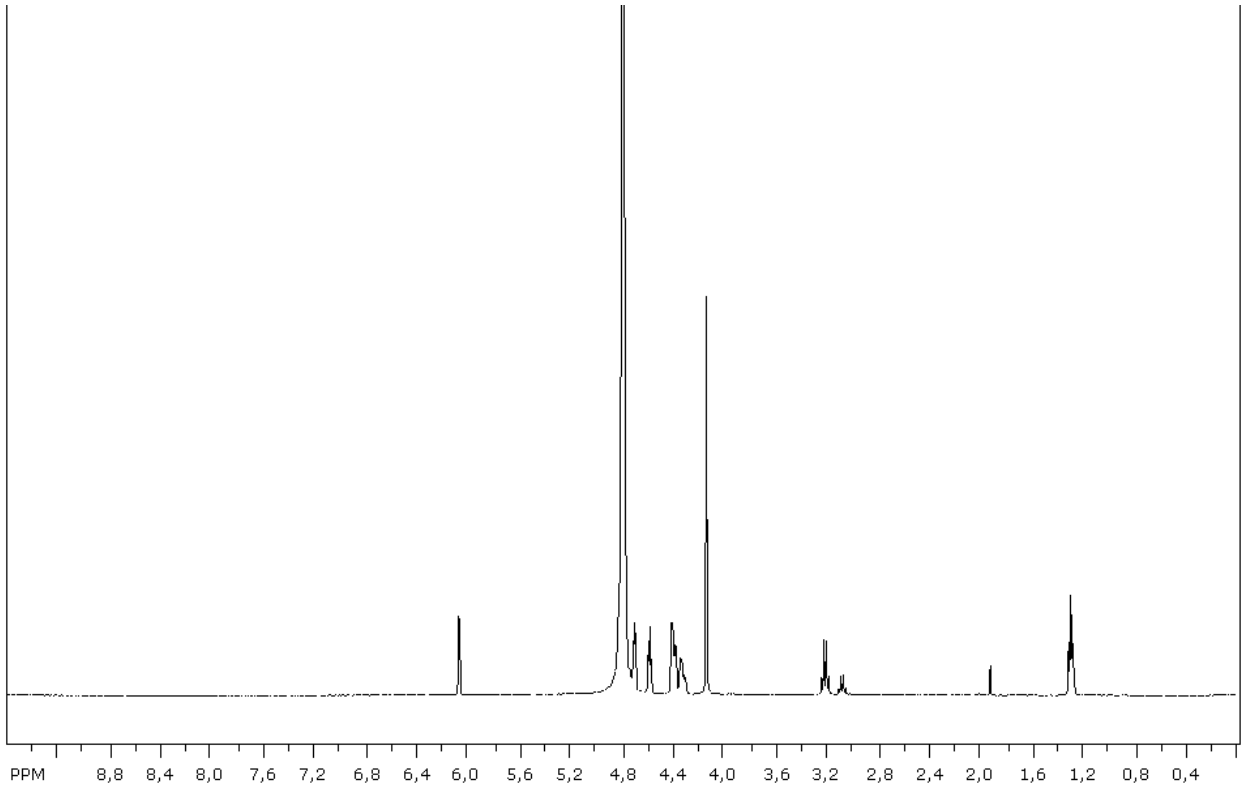
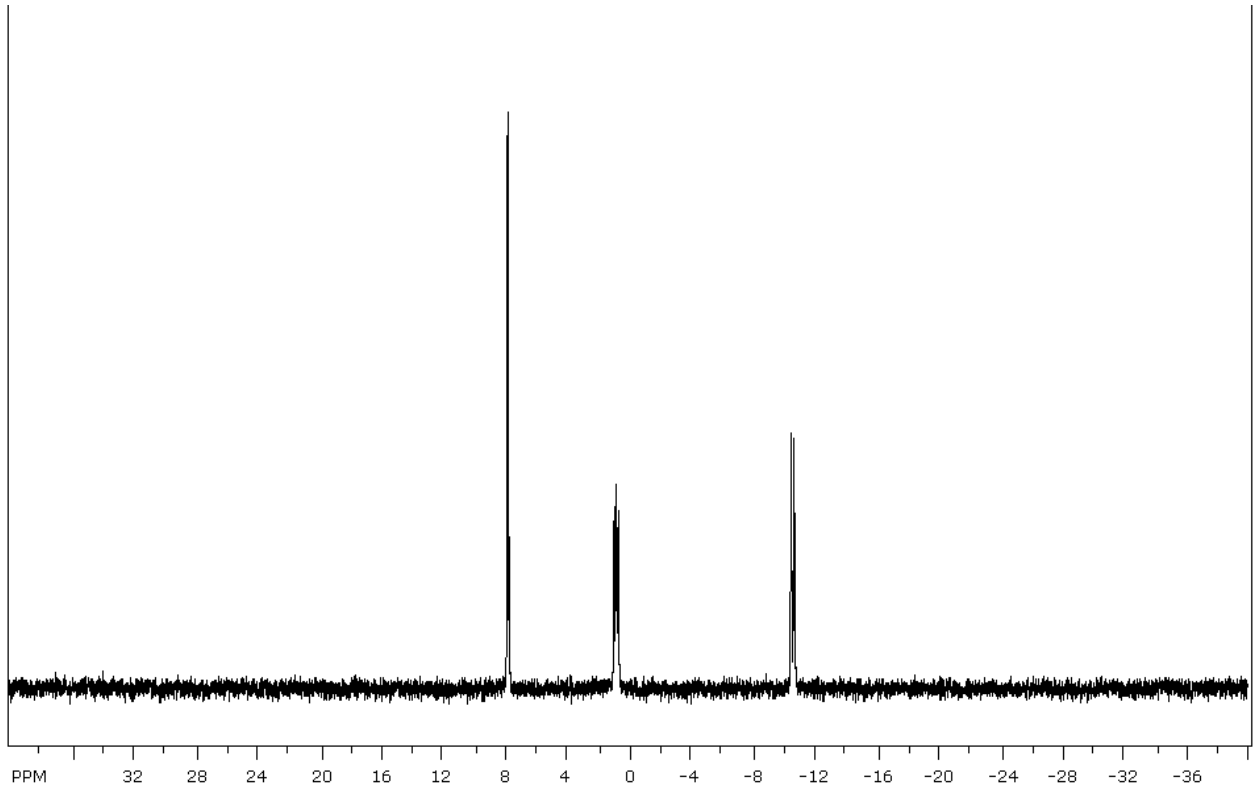


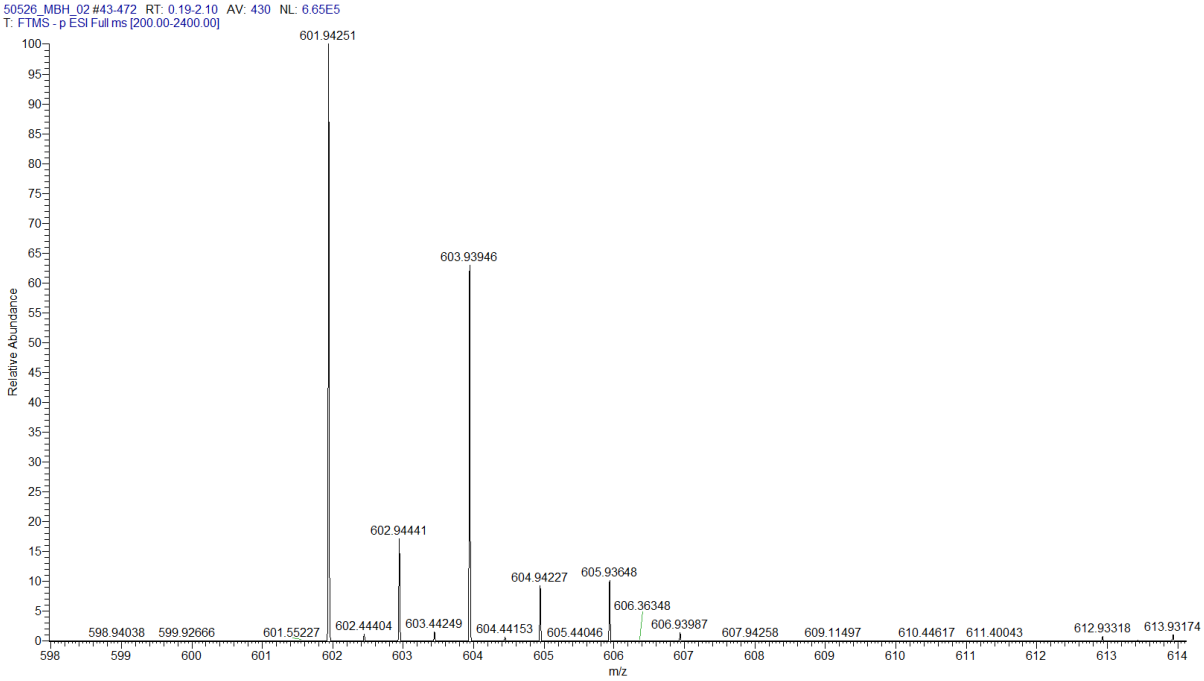
^{1}H NMR spectrum of m^{7}GppCCl$_{2}$p



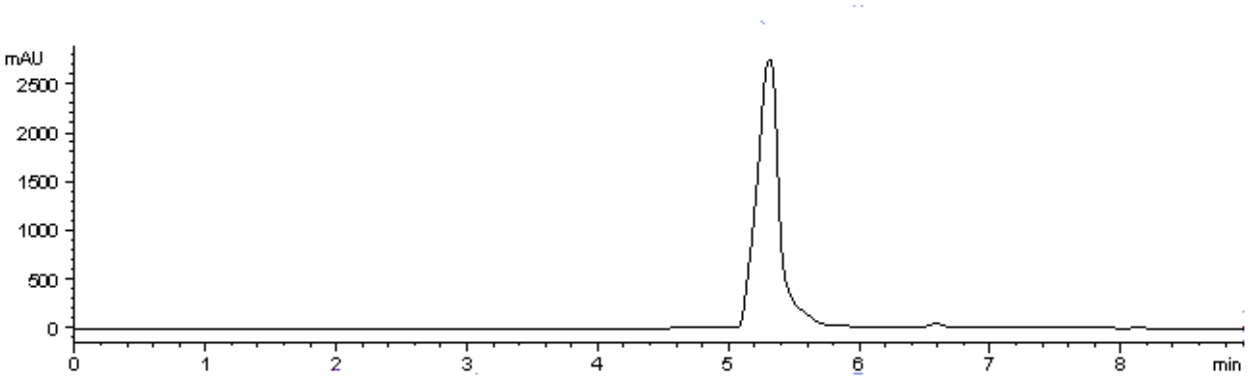
^{31}P NMR spectrum of m^{7}GppCCl$_{2}$p



HRMS spectrum of $m^7GppCCl_2p$



HPLC profile of $m^7GppCCl_2p$



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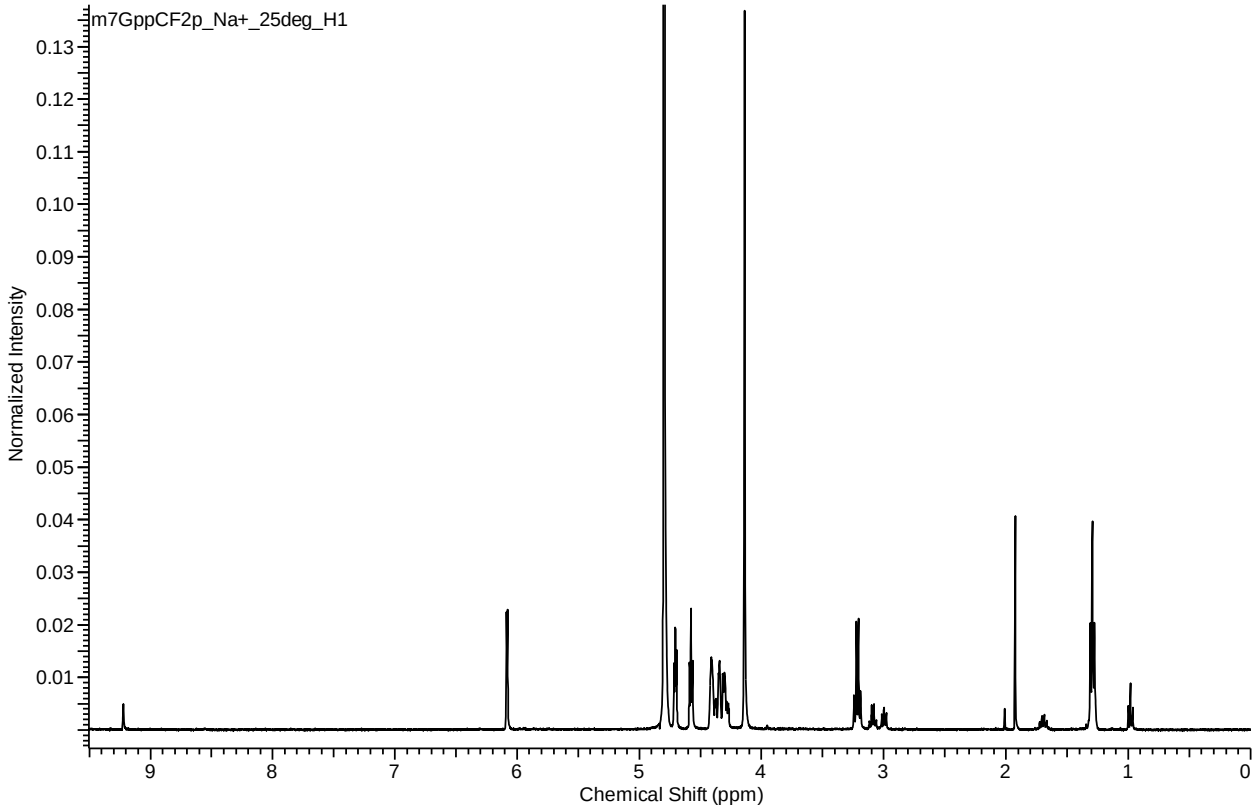
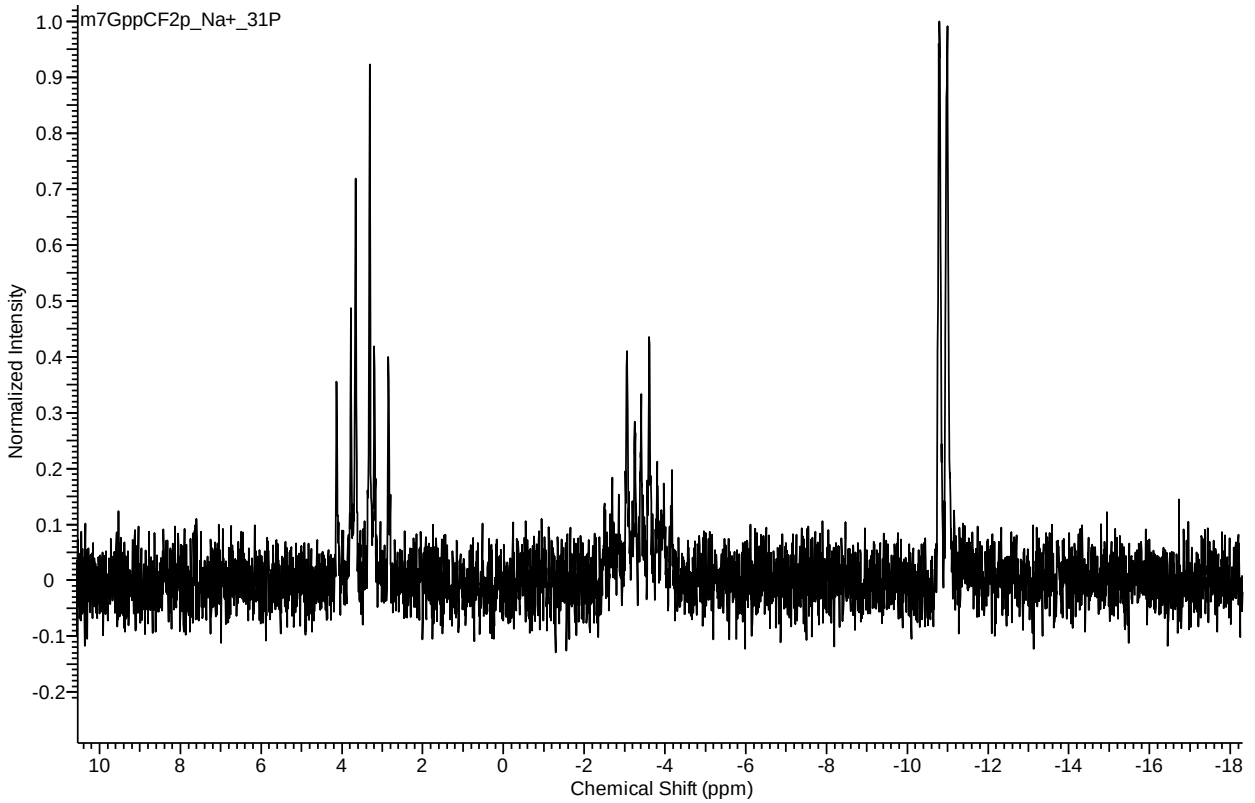
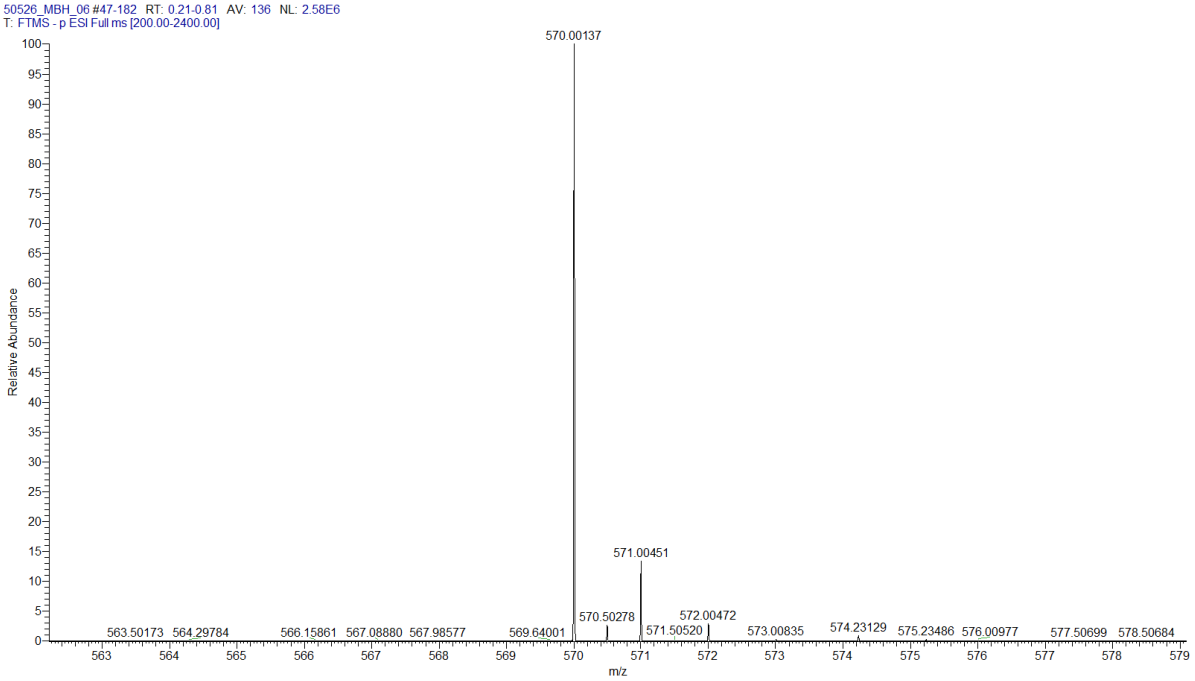


^{31}P NMR spectrum of m^{7}GppCF$_{2}$p



HRMS spectrum of m^7GppCF_2p



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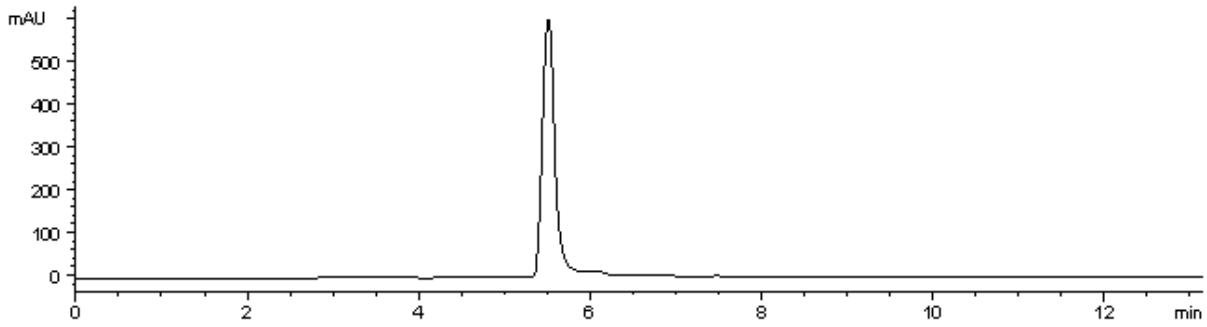

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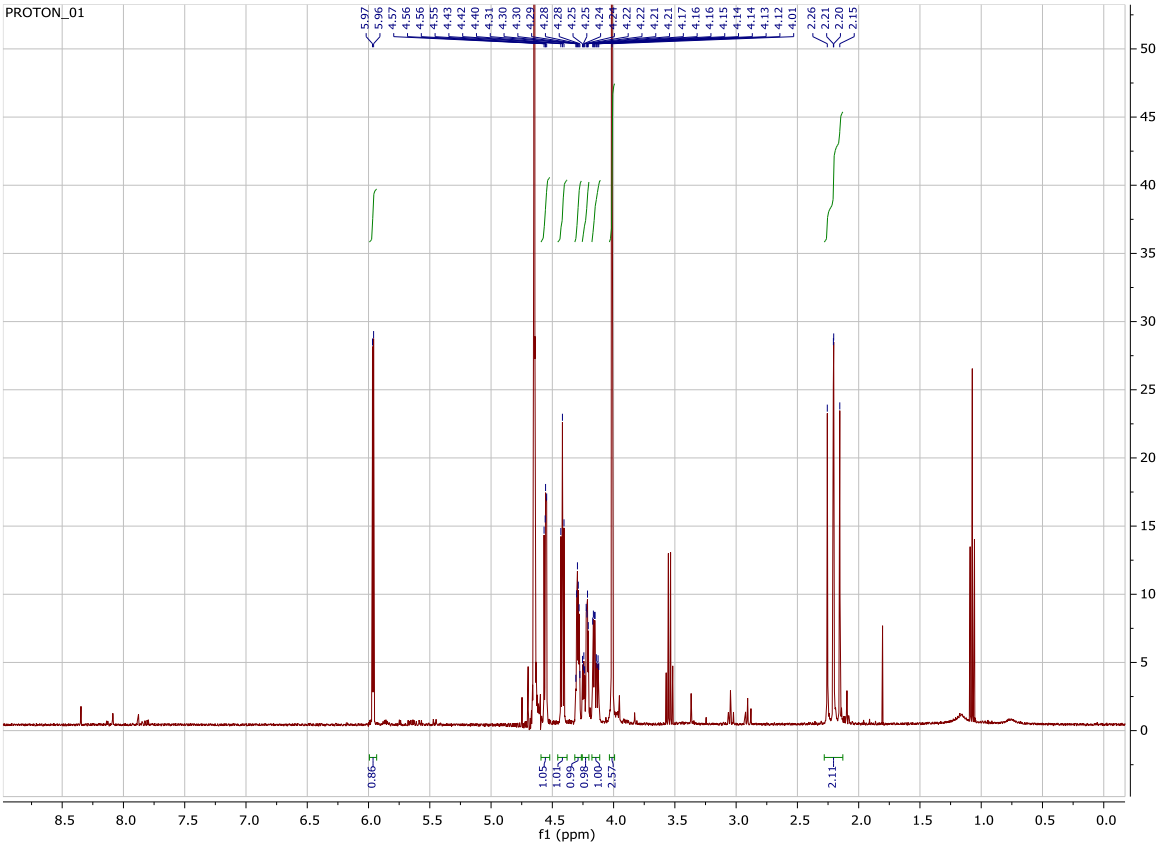

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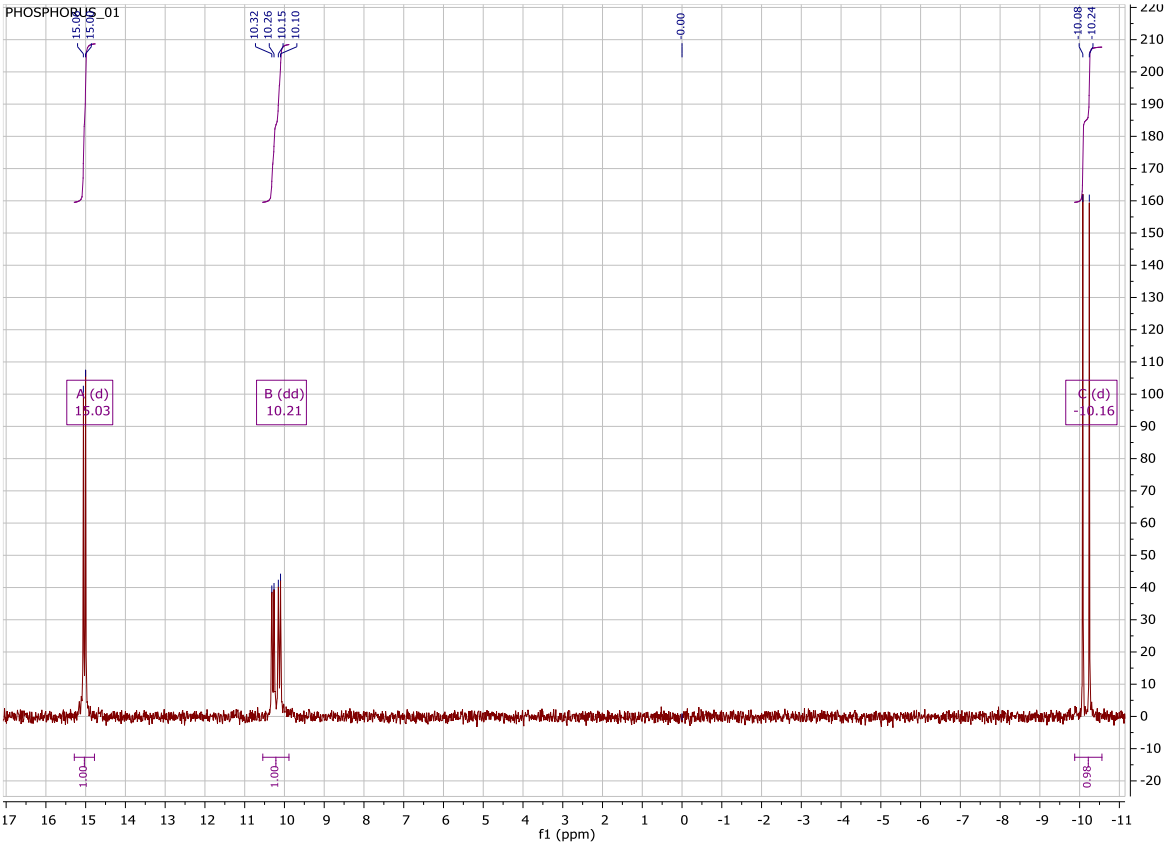

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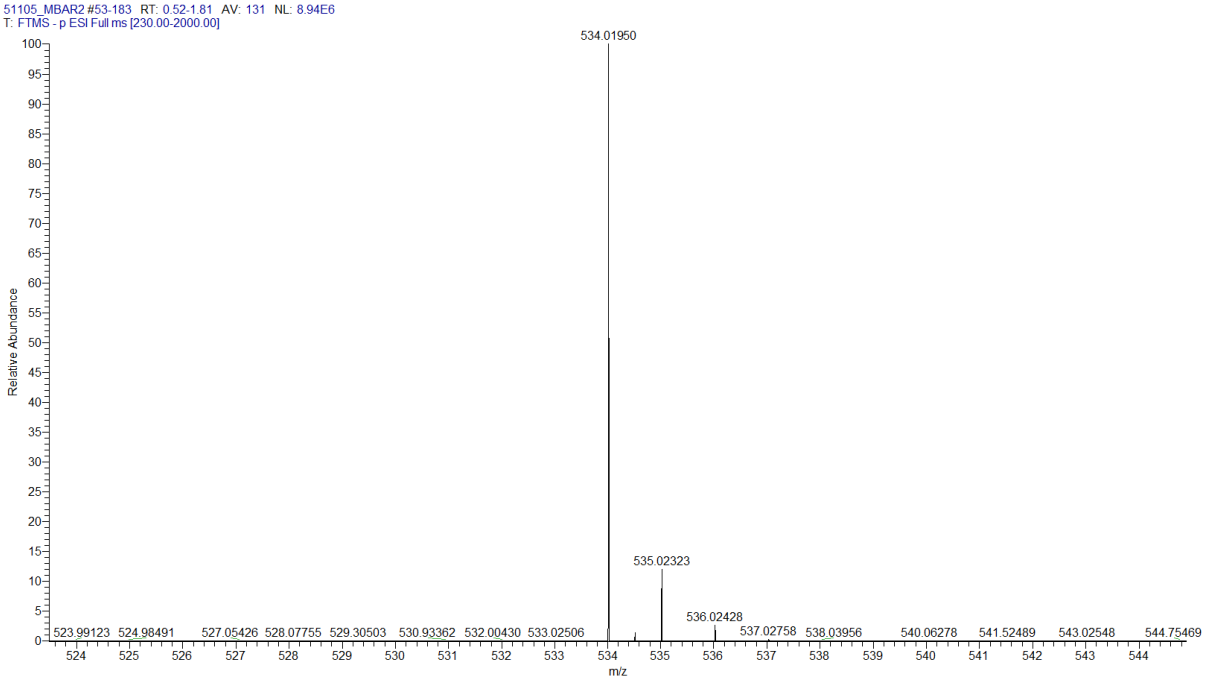

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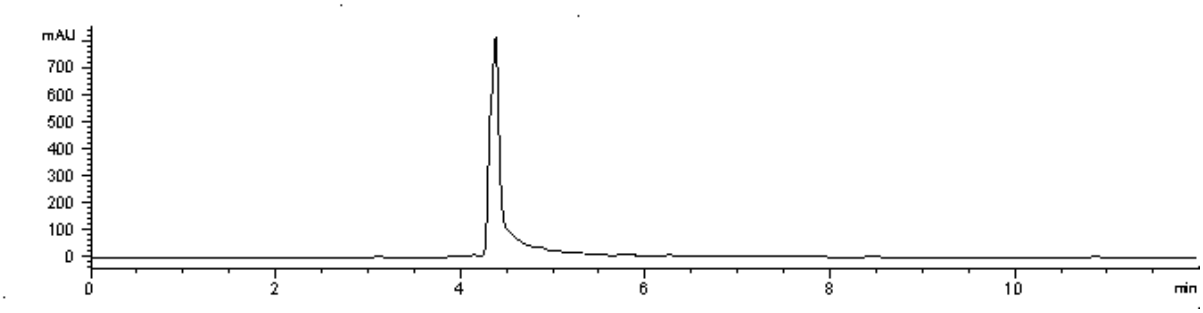
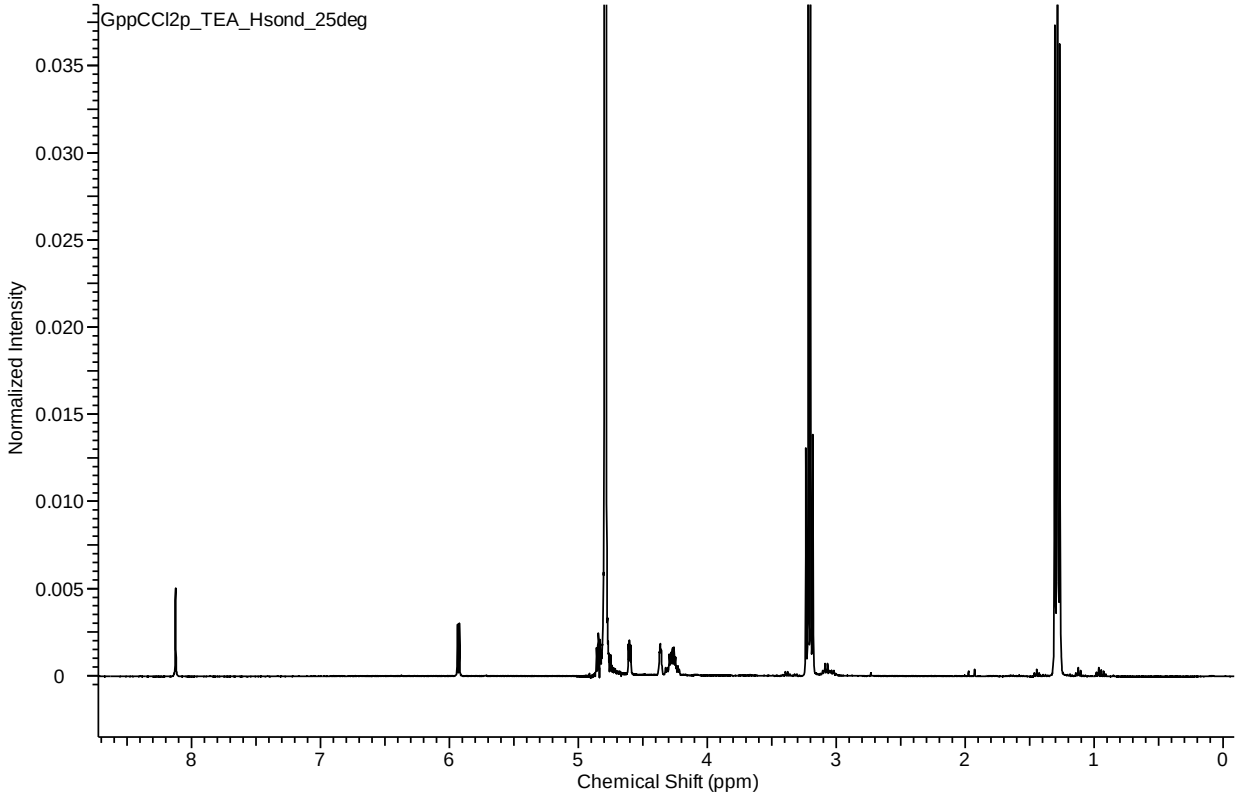
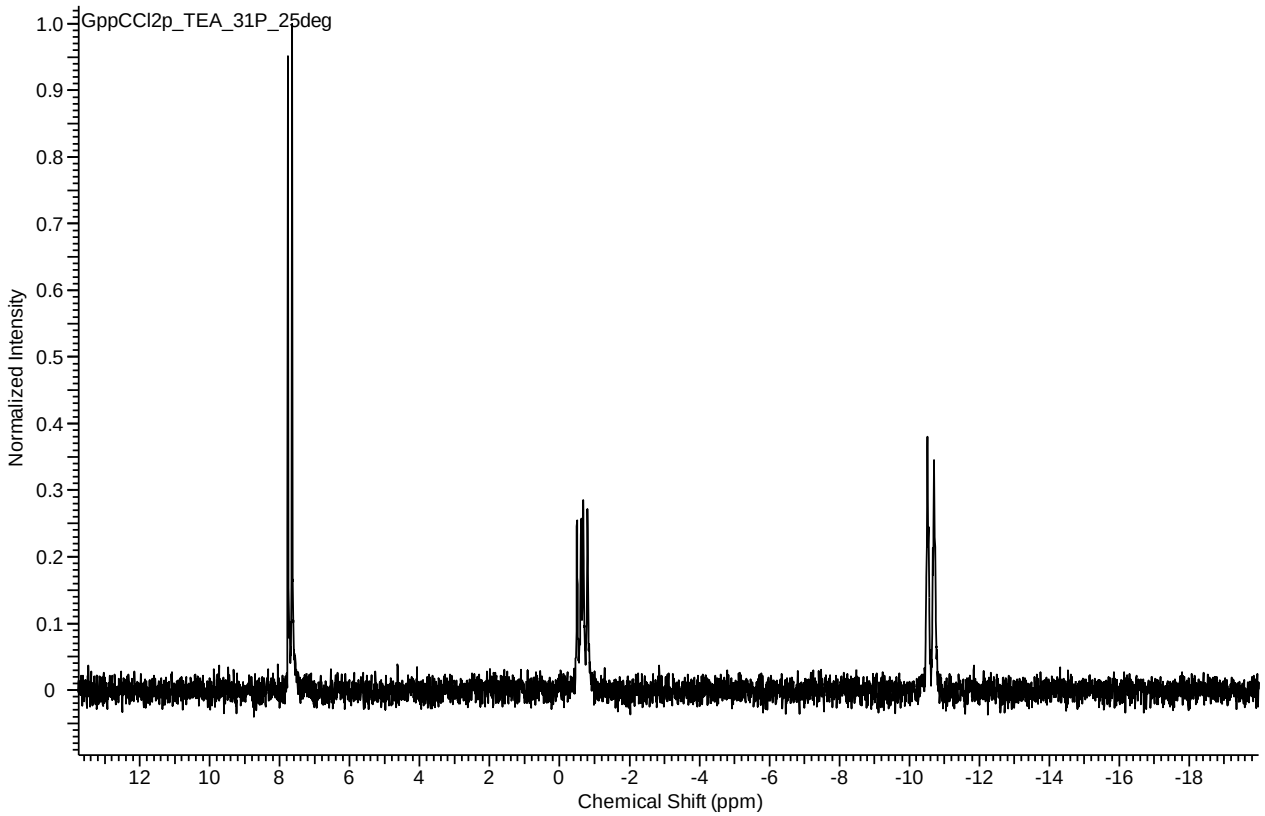


^{1}H NMR spectrum of GppCCl$_2$p



^{31}P NMR spectrum of GppCCl$_2$p



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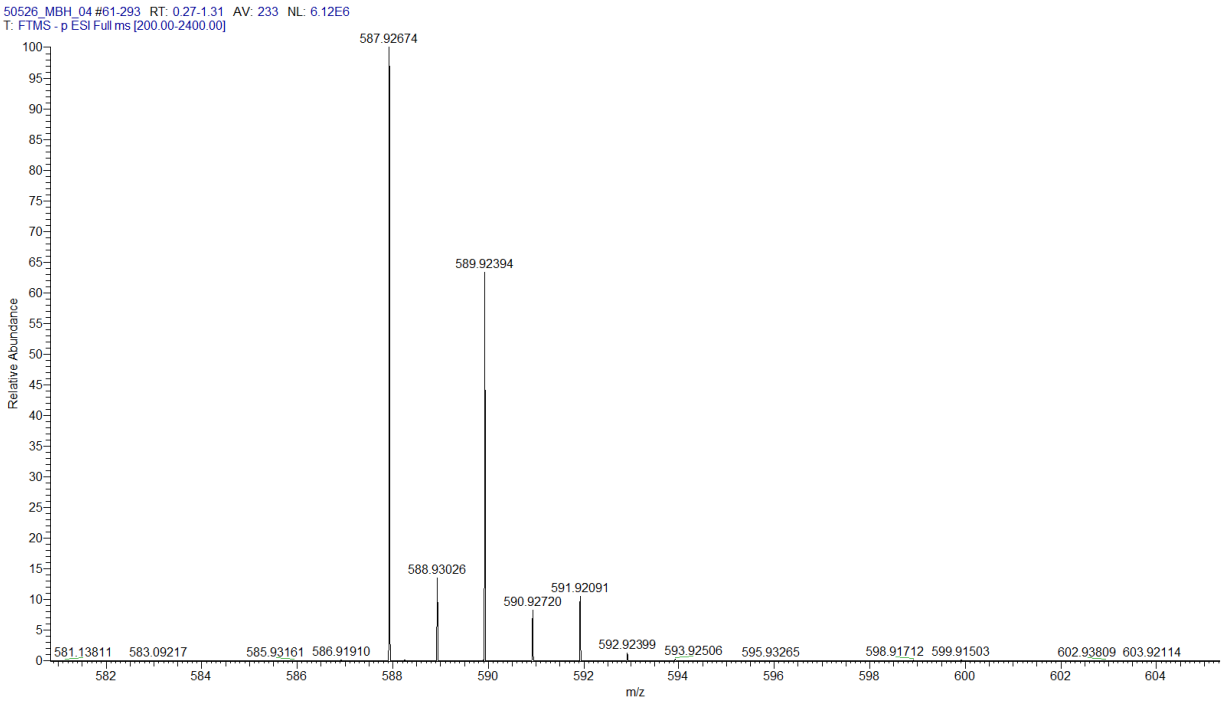

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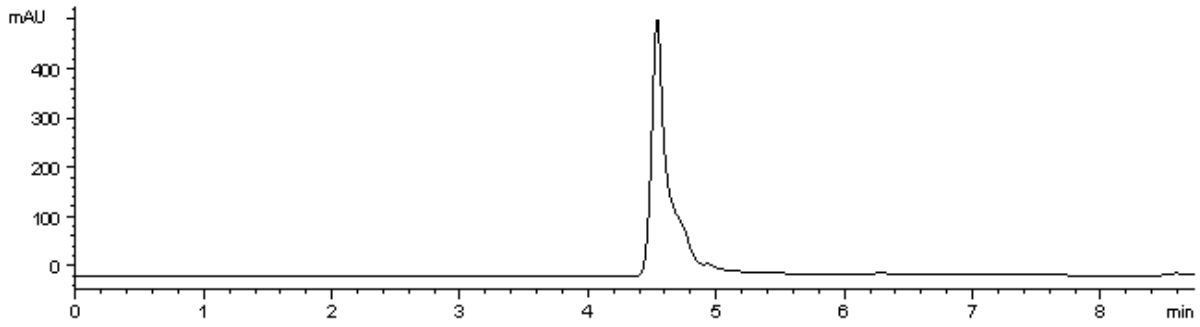
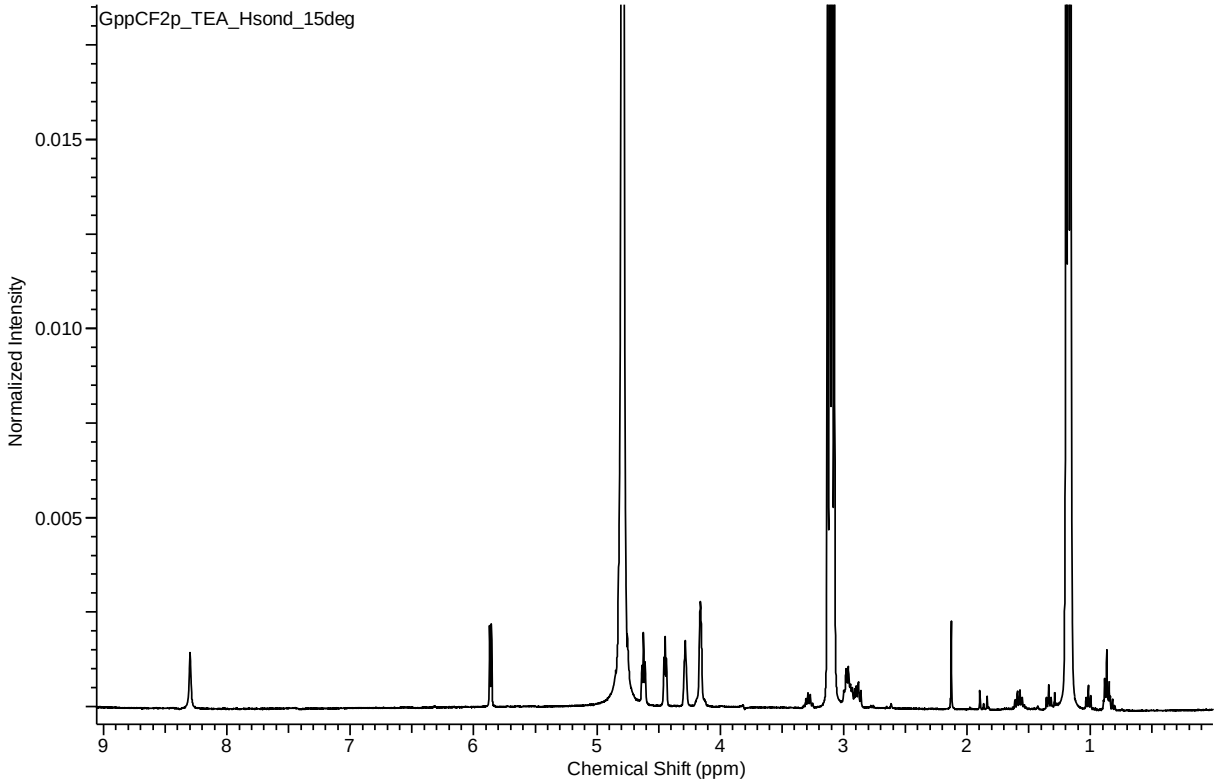
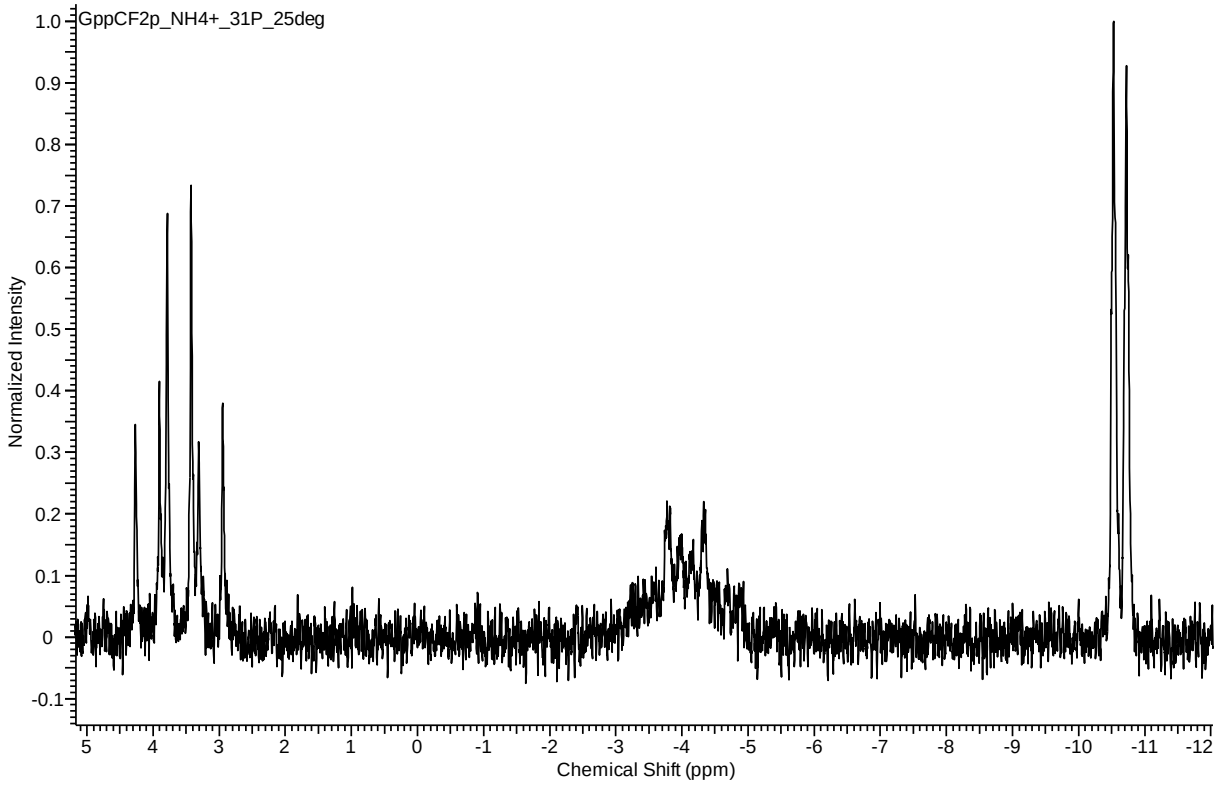


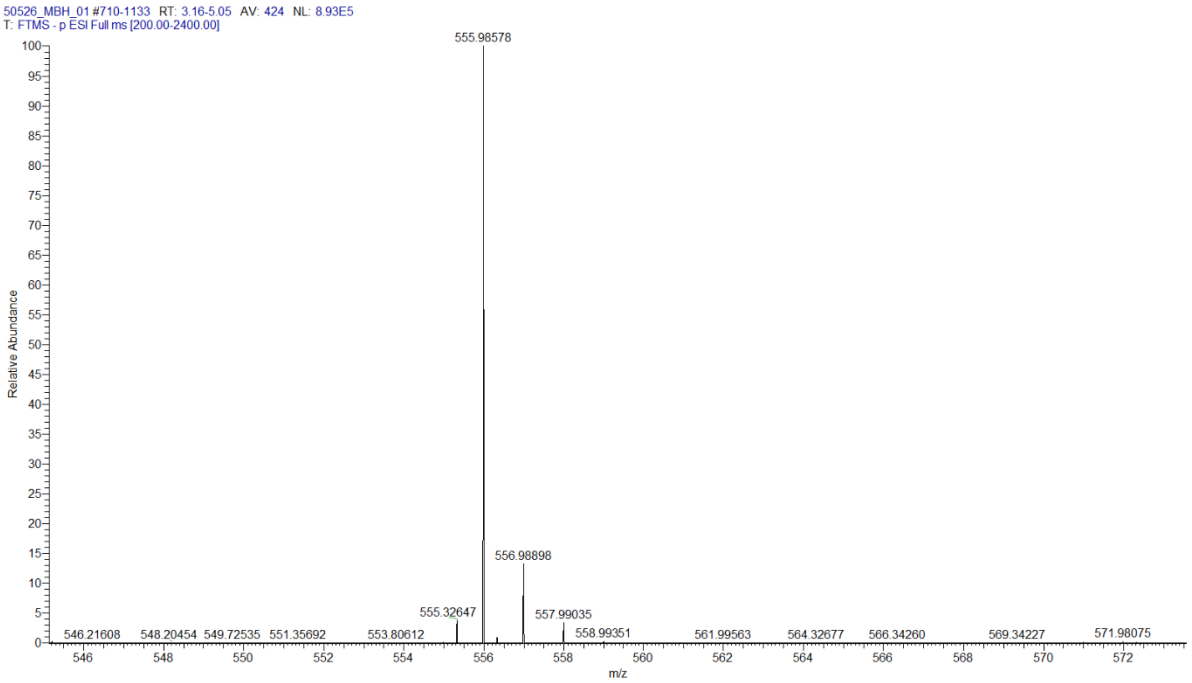
^{1}H NMR spectrum of GppCF$_2$p



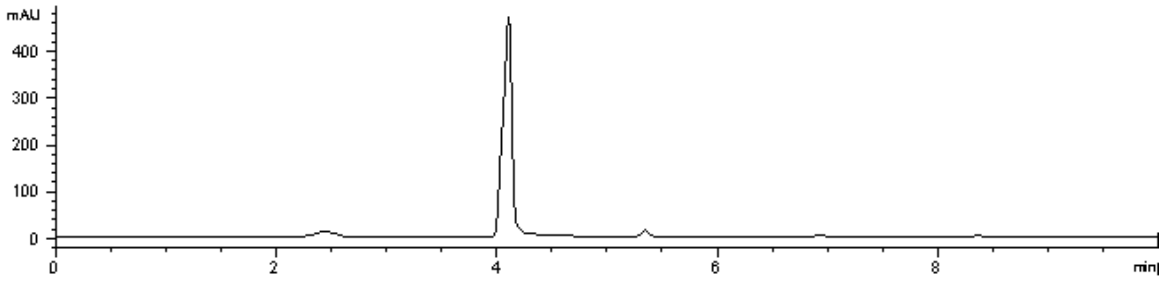
^{31}P NMR spectrum of GppCF$_2$p



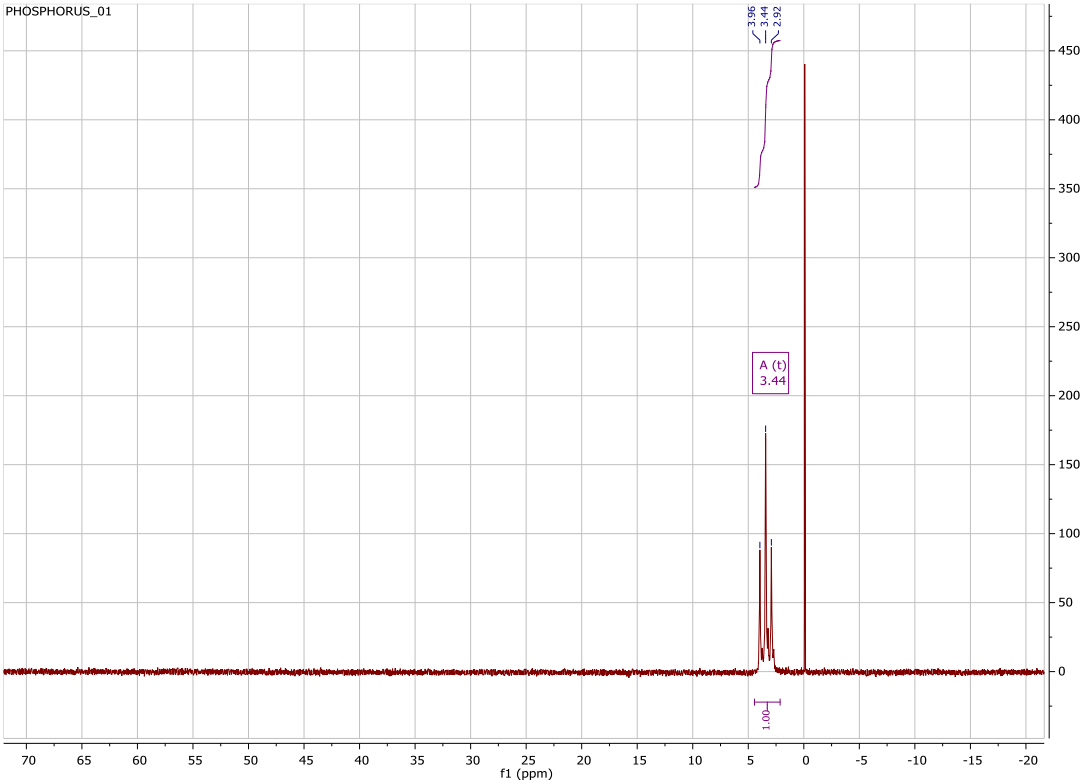
HRMS spectrum of GppCF$_2$p



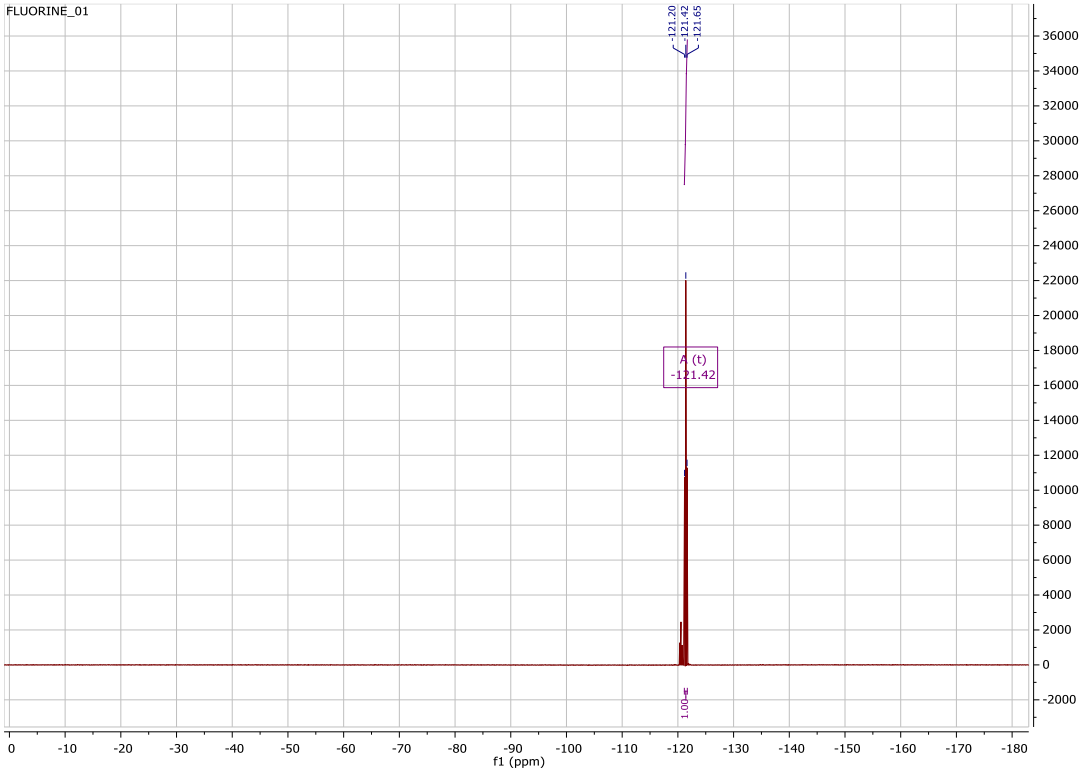
HPLC profile of GppCF$_2$p



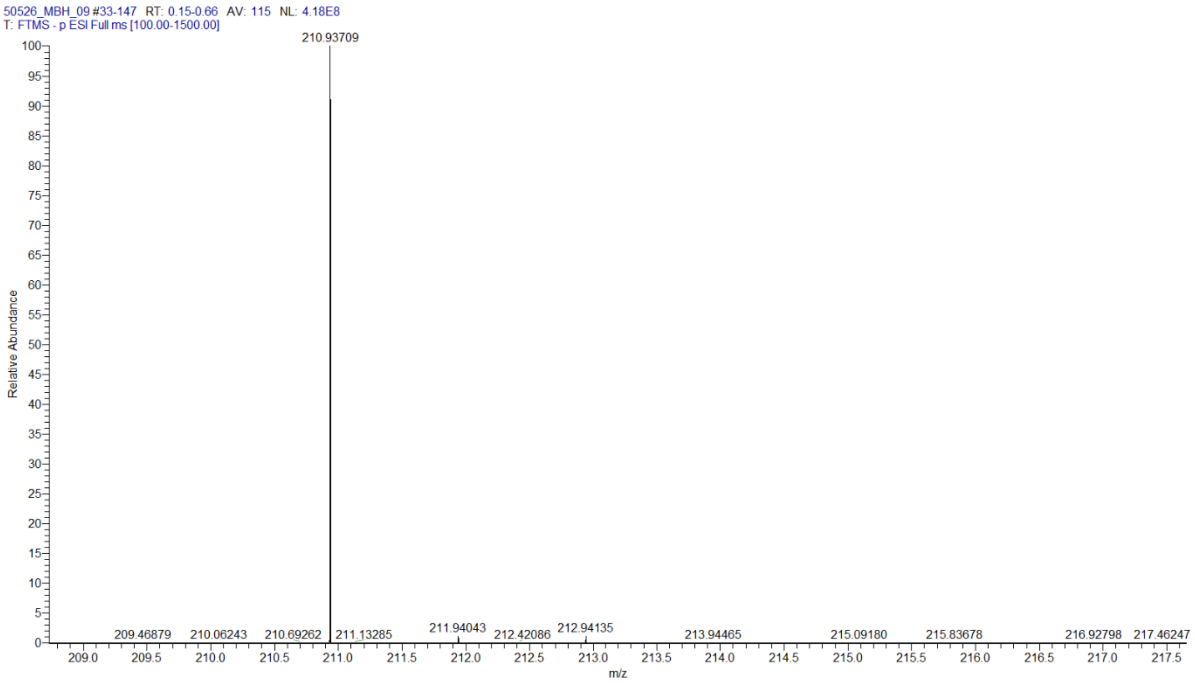
^{31}P NMR spectrum of pCF$_2$p



^{19}F NMR spectrum of pCF$_2$p



HRMS spectrum of pCF_2p



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[1] Blackburn, G. M., Kent, D. E., and Kolkmann, F. (1981) Three new β, γ-methylene analogues of adenosine triphosphate, *J. Chem. Soc. Chem. Comm.*, 1188-1190.

[2] Rydzik, A. M., Lukaszewicz, M., Zuberek, J., Kowalska, J., Darzynkiewicz, Z. M., Darzynkiewicz, E., and Jemielity, J. (2009) Synthetic dinucleotide mRNA cap analogs with tetraphosphate 5',5' bridge containing methylenebis(phosphonate) modification, *Org. Biomol. Chem. 7*, 4763-4776.

[3] Rydzik, A. M., Lukaszewicz, M., Zuberek, J., Kowalska, J., Darzynkiewicz, Z. M., Darzynkiewicz, E., and Jemielity, J. (2009) Synthetic dinucleotide mRNA cap analogs with tetraphosphate 5',5' bridge containing methylenebis(phosphonate) modification, *Org. Biomol. Chem. 7*, 4763-4776.

[4] Jemielity, J., Fowler, T., Zuberek, J., Stepinski, J., Lewdorowicz, M., Niedzwiecka, A., Stolarski, R., Darzynkiewicz, E., and Rhoads, R. E. (2003) Novel "anti-reverse" cap analogs with superior translational properties, *RNA 9*, 1108-1122.

[5] Jemielity, J., Stepinski, J., Jaremko, M., Haber, D., Stolarski, R., Rhoads, R. E., and Darzynkiewicz, E. (2003) Synthesis of Novel mRNA 5′ Cap-Analogues: Dinucleoside P1, P3-Tri-, P1, P4-Tetra-, and P1, P5-Pentaphosphates, *Nucleosides, Nucleotides and Nucleic Acids 22*, 691-694.